THE PREVALENCE AND PUBLIC HEALTH SIGNIFICANCE OF HUMAN PATHOGENIC VIBRIO SPECIES (V. CHOLERAE, V. VULNIFICUS, V. PARAHAEOMOLYTICUS, V. ALGINOLYTICUS) IN HAWAI’I’S DIVERSE TROPICAL COASTAL WATER ENVIRONMENTS

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ABSTRACT

Members of the genus *Vibrio* are ubiquitous and are widely distributed in aquatic environments where they fulfill a major ecological role in the cycling of organic and inorganic compounds in the water. In addition to their role in nature, certain species of *Vibrio* are known to cause opportunistic infections in both aquatic animals as well as humans. The majority of human infections are attributed to four species of *Vibrios*: *V. cholerae*, *V. vulnificus*, *V. parahaemolyticus* and *V. alginolyticus*. These four species cause a variety of infections from mild gastrointestinal infection to septicemia and death. Studies on the prevalence and ecology of *Vibrio* species in tropical areas, such as Hawaii, is limited, and up to now, there have been no studies conducted in Hawaii to determine the prevalence of these pathogens in our coastal waters. The major goals of this study was to determine the prevalence of the four human pathogenic *Vibrio* spp. (*V. cholerae*, *V. vulnificus*, *V. parahaemolyticus*, *V. alginolyticus*) in coastal water environments of Hawaii (islands of Oahu and Hawaii), and to determine the public health significance these pathogens have to people who use these coastal water for recreational purposes. Sampling sites on Oahu included those from beaches impacted and not impacted by land run-off and confined coastal waters (harbors, canals, ponds). Sediments from coastal beaches on Oahu and human sewage were also analyzed for the presence of the four *Vibrio* pathogens. This was to determine if sediments and sewage could be possible sources of contamination of these pathogens into our coastal waters. Sampling sites on the Island of Hawaii included coastal ponds located in the Kona and Hilo areas as well thermal ponds located in Hilo.
The results of this study showed that water salinity and temperature played an important role in the prevalence of *V. vulnificus*, *V. parahaemolyticus*, *V. alginolyticus* and *V. cholerae*. *V. vulnificus* and *V. parahaemolyticus* were prevalent in low salinity sites that were impacted by land run-off but not detectable in high salinity, non-impacted swimming sites. Both species were also prevalent at low salinity swimming ponds on the Island of Hawaii, indicating once again that salinity has a strong influence on the prevalence of these two species. *V. alginolyticus* was prevalent in all sites regardless of salinity. Though this species is generally associated with high salinity waters, nutrients brought in by land run-off can overcome salinity barriers and stimulate *Vibrio* species growth. In addition to low salinity, high water temperature also had an impact on the concentration of the four *Vibrio* spp. pathogens. High temperature, low salinity ponds located on the Island of Hawaii were shown to select for *V. vulnificus*, *V. parahaemolyticus* and *V. alginolyticus*. These ponds have shown past evidence of infection and death due to *V. vulnificus* associated with the use of these ponds. Isolates recovered from these thermal ponds may potentially be more virulent as they have been adapted to survival at temperatures similar to that of human body temperature. *V. cholerae* was not recovered in either impacted or non-impacted sites. Studies have shown that this species is typically found in close association with chitinous organisms, such as zooplankton, during times of environmental stress. The prevalence of pathogenic *Vibrio* spp. in sediments followed a similar trend to what was seen with coastal beach samples. *V. alginolyticus* was prevalent in both primary and secondary beach sediment while *V. vulnificus* and *V. parahaemolyticus* were only prevalent in secondary beach sediment. Thus, based on data from this study it is apparent that sediments from
secondary coastal waters can serve as a source of pathogenic *Vibrio* species into the water column. Data from this study also showed that *V. vulnificus* and *V. parahaemolyticus* were sporadically present in raw and primary treated sewage from three different wastewater treatment plants, while *V. cholerae* was consistently recovered in raw and primary treated sewage from all three treatment plants. Based on this data it is apparent that sporadic shedding of *V. vulnificus* and *V. parahaemolyticus* is occurring within the population, while *V. cholerae* is being consistently shed by asymptomatic carriers. Thus, accidental release of untreated sewage can introduce these pathogens into coastal waters which may lead to public health consequences to users of these waters. Of the four main *Vibrio* spp. pathogens, *V. vulnificus* is capable of causing severe wound infections which can rapidly lead to death. Thus, this species poses a public health significance, and it is therefore recommended that signs be placed in areas where this species is prevalent warning users of the potential for infection.

In summary, data gathered from this study was able to provide basic information, that was lacking, regarding the distribution of the four main human *Vibrio* pathogens in a tropical area such as Hawaii. This data was then used to make a basic assessment of the potential public health significance these pathogens have on humans who use Hawaii’s coastal waters for recreational purposes, and to determine if and when warning signs would be warranted to notify the public of the potential risk for infection.
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CHAPTER 1
REVIEW OF LITERATURE: VIBRIO BACTERIA

1.1. CLASSIFICATION AND GENERAL PROPERTIES OF VIBRIO SPECIES (SPP.)

The word vibrio means comma shaped and is the basic structure of the group of bacteria represented by Vibrio cholerae, a bacterial pathogen of man that has been considered one of the scourges of mankind. Today, the word “vibrio” is a common term used in lay, as well as scientific literature, to refer to a group of bacteria that can cause diseases in many animals. The scientific community uses the binomial nomenclature to identify each living thing on earth to its genus and species. Using this system the genus Vibrio belongs to the family Vibrionaceae, which includes many genera, such as Allomonas, Enhydrobacter, Listonella, Photobacterium and Salinivibrio (Boone and Castenholz, 2001). The genus Vibrio is characterized as comma shaped, gram-negative, facultative anaerobic bacteria, that contain one flagella. Other properties of bacteria belonging to the genus Vibrio are, production of oxidase and catalase and fermentation of glucose without the production of gas. Most Vibrio spp. are halophilic, requiring sodium for growth, and are considered a marine bacteria. The primary habitats of Vibrio spp. are marine waters, including estuaries, marine coastal waters as well as man-made aquaculture settings used for food production. In these habitats Vibrio spp. colonize surfaces of sediments, rocks, walls, piers, bottoms of boats, plants, and marine animals as well as zooplankton. However, some species (e.g. V. cholerae) have been recovered from fresh water environments. Currently there are more than 65 known species of Vibrio with many environmental strains yet to be speciated. Recent improvements in the taxonomy of bacteria using genomic techniques (i.e, DNA-DNA hybridization, DNA
nucleotide composition, 16S RNA sequencing) (Thompson and Swings, 2006) have resulted in the description of new *Vibrio* species.

Recently an American group (Heidelberg et al. 2000) and a Japanese group (Yamaichi et al., 1999) independently reported that members of the genus *Vibrio* carry two circular chromosomes as compared to most bacteria which carry a single chromosome. Pulse-field gel electrophoresis (PFGE) of undigested genomic DNA from purified bacteria revealed the presence of two bands as well as the presence of housekeeping genes on both replicons, which indicated that they were chromosomes rather than megaplasmids (Iida and Kurokawa, 2006). Most genes required for growth are contained on the larger of the two chromosomes (Heidelberg et al., 2000) while the smaller chromosome contains most genes needed for bacterial adaptation to environmental changes (Makino et al., 2003). Initially the two chromosome arrangement was discovered in *V. parahaemolyticus* but was subsequently found in other *Vibrio* species such as *V. vulnificus*, *V. fluvialis* and *V. cholerae* as well as other members of the *Vibrionaceae* family (Iida and Kurokawa, 2006). It has been suggested that having two chromosomes may be advantageous to *Vibrio* spp. during DNA replication (Yamaichi et al., 1999), which would account for some of the more rapid rates of replication in some species (i.e. 8 to 9 minute doubling time in *V. parahaemolyticus*) (Joseph et al., 1982).

### 1.2. ECOLOGICAL ROLES OF VIBRIO SPP.

The basic ecological roles of microorganisms in terrestrial and aquatic environments is to degrade everything that enters that environment to basic chemical components. The four basic chemicals required for the growth of all organic forms of life are carbon, nitrogen, sulfur and phosphorous. In water environments, dissolved organic
matter (DOM) describes organic matter in a dissolved form that must be further metabolized to release the four basic chemicals in a form that can be utilized for growth by all living organisms, particularly microorganisms. In marine environments, a heterogenous group of bacteria, called heterotrophic bacteria, have been identified with the capacity to further degrade DOM into the basic nutrients (carbon, nitrogen, phosphorus, sulfur). *Vibrio* spp. are one group of heterotrophic bacteria that contribute to nutrient recycling within the diverse habitats they occupy (Thompson and Polz, 2006).

Similar to other gram negative bacteria, *Vibrio* species degrade complex organic and inorganic matter in a sequential manner by respiratory or fermentative metabolism. The oceans, the largest reservoir of biologically active carbon, contain about $10^{13}$ tons of carbon. Heterotrophic organisms, like *Vibrio* spp., provide a sink for fixed carbon through assimilation of DOM and remineralization as carbon dioxide ($CO_2$), for use by higher tropic levels (Munn, 2004). Thompson and Polz reported that complex polymers are partially hydrolyzed by extracellular enzymes produced by *Vibrio* spp. (Thompson and Polz 2006). These hydrolyzed by-products are then transported into the periplasmic space of *Vibrio* spp. and used as nutrients. Other microorganisms also use these by-products as sources of nutrients, making *Vibrio* spp. important players in the marine food web. Figure 1.1 depicts the role microbes, such as *Vibrio* spp., play in the degradation of complex DOM into nutrients (carbon, nitrogen, phosphorus, sulfur) that can be used by organisms in higher tropic levels for their growth and metabolism.

Some *Vibrio* spp. are also known to play a role in the cycling of marine nitrogen by fixation of nitrogen gas ($N_2$) to organic nitrogen, the reduction of nitrate to nitrite or conversion of organic nitrogen to ammonia. In this regard, estuaries are frequently
nitrogen limited, making the ability to reduce atmospheric nitrogen to ammonia highly advantageous (Criminger et al., 2007). Breakdown of organic materials by microbes and zooplankton provide nitrogen to members of nitrogen limited ecosystems. Nitrogen, in its various forms, is essential to all life forms in the synthesis of proteins and nucleic acids. Phosphorus, another nutrient essential to life, is required for biological processes such as membrane phospholipid synthesis, signaling pathways and energy metabolism. *Vibrio* spp. express a number of extracellular enzymes (i.e. alkaline phosphotases) that degrade phosphorus-containing macromolecules, making them available for primary production (Thompson and Polz, 2006). Thus, by the degradation of DOM into more

Figure 1.1. The microbial loop depicting the degradation of dissolved organic matter (DOM) by microbes in the ocean, making nutrients such as carbon, nitrogen, phosphorus and sulfur which can then be used by organisms of higher tropic levels (taken from http://sites.google.com/site/ashvinichauhan/).
simple and usable carbon, nitrogen and phosphorus compounds in the marine environment, *Vibrio* species play an essential role in the microbial food web by making these compounds available for use by other members of the ecosystem.

A specific role *Vibrio* spp. plays in the environment is in the degradation of chitin which is a component of the exoskeleton of many marine organisms such as cuttlefish, crab, lobster, zooplankton and algae (Riemann and Azam, 2002). Chitin, a homopolymer of $N$-acetylglucosamine (NAG), is one of the most abundant biopolymers in nature, and is produced in massive quantities in the marine environment where it is rapidly catabolized by marine bacteria. More than $10^{11}$ tons of chitin are estimated to be produced annually in marine waters alone, mostly from copepods. Marine ecosystems would be inundated with this highly insoluble polysaccharide were it not for chitinivorous bacteria capable of degrading and converting it to biologically useful substances (Li et al., 2007). The degradation of these large quantities of chitin is critical for maintaining the carbon and nitrogen cycles in marine waters (Li and Roseman, 2004). Chitinase activity may reflect one of the most important extracellular enzymatic processes in the marine environment (Thompson and Polz, 2006), and several marine bacteria, including most *Vibrio* spp., are capable of chitinase activity. The breakdown of chitin involves many genes and enzymes and is a sequential process. Initially, bacterial cells bind to chitin and secrete chitinases. The partially hydrolyzed chitin oligosaccharides (chitooligosaccharides) are then transported into the periplasmic space of bacterial cells via a specific chitooligosaccharide porin. The chitooligosaccharides are then further hydrolyzed by enzymes in the periplasmic space and translocated into the cytoplasm by specific transporters, where they are catabolized into fructose-6-phosphate,
ammonia and acetate. These compounds are then used by cells for metabolism and growth. The association between *V. cholerae* and chitinous copepods has been known to be an important part of the life cycle of *V. cholerae* and its ability to infect humans (Li et al., 2007) (Figure 1.2). This is because chitin resists digestion by acid, allowing *V. cholerae* to survive gastric transit through the adhesion to ingested chitin particles (Nalin et al., 1979). During spring and late summer in Bangladesh, zooplankton blooms occur followed by outbreaks of cholera. Studies have shown that a single copepod can carry up to $10^4$ cells of *V. cholerae* (Huq et al., 1983; Heidelberg et al., 2002). Thus, copepod blooms can result in concentrations of *V. cholerae* capable of causing infection. Ponds and rivers are often used as sources of drinking water in Bangladesh and the filtering of
water using sari cloth is a simple method that has shown to significantly remove copepods, and thus reduce cholera rates in this country (Colwell et al., 2003).

In addition to the cycling of nutrients and their association with copepods, *Vibrio* spp. can also form symbiotic relationships with marine animals (Table 1.1). One well

Table 1.1. List of representative *Vibrio* spp. and their functional roles.

<table>
<thead>
<tr>
<th><em>Vibrio</em> Species</th>
<th>Role</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>V. aestuarianus</em></td>
<td>Infection in Pacific oyster <em>Crassostrea gigas</em>¹</td>
</tr>
<tr>
<td><em>V. anguillarum</em></td>
<td>Marine fish pathogen²</td>
</tr>
<tr>
<td><em>V. chagasii</em></td>
<td>Fish pathogen³</td>
</tr>
<tr>
<td><em>V. coralliilyticus</em></td>
<td>A pathogen of coral, fish and shellfish⁴</td>
</tr>
<tr>
<td><em>V. fischeri</em></td>
<td>Symbiont of squid, luminescent bacterium⁷</td>
</tr>
<tr>
<td><em>V. gallicus</em></td>
<td>Symbiont of abalone⁵</td>
</tr>
<tr>
<td><em>V. halioticoli</em></td>
<td>Symbiont of abalone⁶</td>
</tr>
<tr>
<td><em>V. ichthyoenteri</em></td>
<td>A pathogen of Japanese flounder²</td>
</tr>
<tr>
<td><em>V. mediterranei</em></td>
<td>A coral pathogen⁴</td>
</tr>
<tr>
<td><em>V. natriegens</em></td>
<td>Certain strains can cause gastroenteritis in humans²</td>
</tr>
<tr>
<td><em>V. ordalii</em></td>
<td>A pathogen of wild and cultured salmon⁴</td>
</tr>
<tr>
<td><em>V. salmonicida</em></td>
<td>A marine fish pathogen²</td>
</tr>
<tr>
<td><em>V. shilo</em></td>
<td>A coral pathogen²</td>
</tr>
<tr>
<td><em>V. splendidus</em></td>
<td>Mutualistic, opportunistic, and pathogenic for marine animals⁴</td>
</tr>
<tr>
<td><em>V. superstes</em></td>
<td>Symbiont of abalone⁵</td>
</tr>
<tr>
<td><em>V. alginolyticus</em></td>
<td>Wound infections in humans²</td>
</tr>
<tr>
<td><em>V. cholerae</em></td>
<td>Ecological agent of cholera in humans⁷</td>
</tr>
<tr>
<td><em>V. cincinnatiensis</em></td>
<td>Gastroenteritis and wound infections in humans⁶</td>
</tr>
<tr>
<td><em>V. damselae</em></td>
<td>Wound infections in humans⁶</td>
</tr>
<tr>
<td><em>V. fluvialis</em></td>
<td>Gastroenteritis in humans⁶</td>
</tr>
<tr>
<td><em>V. furnissii</em></td>
<td>Gastroenteritis in humans⁶</td>
</tr>
<tr>
<td><em>V. harveyi</em></td>
<td>Wound infections in humans⁶</td>
</tr>
<tr>
<td><em>V. hollisae</em></td>
<td>Gastroenteritis in humans⁶</td>
</tr>
<tr>
<td><em>V. metschnikovii</em></td>
<td>Gastroenteritis in humans⁶</td>
</tr>
<tr>
<td><em>V. mimicus</em></td>
<td>Gastroenteritis in humans⁶</td>
</tr>
<tr>
<td><em>V. parahaemolyticus</em></td>
<td>Gastroenteritis in humans⁶</td>
</tr>
<tr>
<td><em>V. vulnificus</em></td>
<td>Gastroenteritis, wound infections and septicemia in humans²</td>
</tr>
</tbody>
</table>

¹Saulnier et al 2009  
²Rowe-Magnus et al 2006  
³Austin et al 2005  
⁴Thompson et al 2005  
⁵Sawabe et al 2004  
⁶Nishibuchi 2006
known relationship is that between *V. fischeri* and the Hawaiian bobtail squid (*Euprymna scolopes*). This symbiosis has been well studied and serves as a model for many animal-bacteria interactions. *V. fischeri*, from the environment, colonizes the light organ of juvenile *E. scolopes*, and it is the bioluminescent capacity of this *Vibrio* species that produces light, which is used by the squid as an antipredatory mechanism. The light organ of the squid functions in the camouflaging behavior known as counterillumination, wherein, the squid emits light downward, matching the light from above and obscuring their silhouette from predators beneath them (Harper and Case, 1999). In turn, *V. fischeri* cells receive a nutrient rich environment safe from predators. Because the light organ is nutrient rich *V. fischeri* populations can reach >10^5 cells within hours, and can achieve generation times of 20-30 minutes, rivaling the maximum growth rate typically observed in culture (Ruby and Asato, 1993).

A second example of a symbiosis between *Vibrio* spp. and a marine organism is that between *V. halioticoli* and abalones (*Haliotis* species). *V. halioticoli* is unusual in that it is a non-flagellated, non-motile *Vibrio* species. These unusual properties of this *Vibrio* sp. probably reflects the loss of these attributes because the abalone gut provides adequate nutrients which allow *V. halioticoli* to grow to high concentrations (10^6-10^8 cells per gram) (Sawabe et al., 1995). This *Vibrio* sp. constitutes approximately 70% of the viable bacterial population in the abalone gut and is believed to aid the abalone in its digestion of algae. In this regard, *V. halioticoli* has been reported to produce multiple alginate degrading enzymes that degrade alginates, which are the structural components of marine brown algae (Draget et al., 2005). In addition, *V. halioticoli* strains may be involved in the cooperative degradation of seaweed polysaccharides (Sawabe et al., 1995).
and are capable of fermenting energy-rich carbohydrates into volatile short-chained fatty acids that can be used by the abalone as a source of energy (Sawabe, 2006).

1.3. *VIBRIO* SPP. AS PATHOGENS OF CORAL

Coral reefs are found in warm, shallow waters of tropical oceans and are among the most diverse and productive ecosystems in the world. They are productive owing to the successful symbiosis between the coral animal (polyps) and endosymbiotic algae, known as zooxanthellae (Rosenberg and Koren, 2006). Coral polyps produce calcium carbonate which forms the skeleton of coral reefs, and a single coral colony can consist of thousands of coral polyps. The zooxanthellae live in symbiosis within the tissue of coral polyps and provide nutrients via photosynthesis. Coral bleaching occurs when the zooxanthellae die and the coral loses its pigment, leading to a bleached, white appearance. It has been suggested that coral bleaching is triggered by environmental factors, such as increased seawater temperature, that impose stress on coral (Banin et al., 2000). The zooxanthellae generally have a mechanism to repair photodamage but when water temperature is too high, proteins that repair damage are denatured and are ineffective in repairing the damage (Rosenberg and Koren, 2006).

*V. shiloi* is one species of *Vibrio* that is known to cause bleaching of coral. Infection and bleaching of coral, *Oculina patagonica*, has been shown to be water temperature dependent, occurring only when seawater temperatures exceed 20°C (Kushmaro et al., 1998). During infection, *V. shiloi* adheres to the surface of *O. patagonica* and penetrates into the epidermis of its coral host. Once inside the coral, the bacteria multiply and produce extracellular, heat-resistant and heat-sensitive toxins that lyse zooxanthellae, inhibiting photosynthesis and resulting in coral bleaching (Banin et
al., 2000). It has been shown experimentally that infection of *O. patagonica* by *V. shiloi* is temperature dependent. Bacterial cells grown at 25°C were able to adhere to coral and initiate infection, whereas cells grown at 16°C did not adhere to coral (Kushmaro et al., 1998). Furthermore, virulence determinants of *V. shiloi* were shown to be expressed only when water temperatures exceeded 25°C. At 29°C bleaching is rapid and complete; at 25°C, it is slower and incomplete; and below 20°C, no bleaching occurs even with a very high inoculum size of *V. shiloi* (Rosenberg and Koren, 2006).

A second *Vibrio* species, *V. coralliilyticus*, causes bleaching as well as death of the coral species *Pocillopora damicornis* in the Indian Ocean and Red Sea. Similar to *V. shiloi* infection, *V. coralliilyticus* infection is temperature dependent. At water temperatures below 22°C no infection occurs while at 24 to 26°C infection results in bleaching of coral. At water temperatures between 27 and 29°C, infection causes rapid tissue lysis and death of the coral (Rosenberg and Koren, 2006). In summary, elevated water temperatures enhance coral infection by *V. coralliilyticus*, which can cause coral bleaching by killing intracellular zooxanthellae, and can lead to coral death by production of extracellular proteases that lyse coral tissue (Ben-Haim et al., 2003).

1.4. **VIBRIO** SPP. AS PATHOGENS OF FISH AND OTHER MARINE ANIMALS

*Vibrio* spp. can cause opportunistic infections of fish (Reed and Francis-Floyd, 1996; Fouz et al., 2002) in marine and estuarine environments throughout the world. In addition, vibrio infection can cause significant mortalities of fish in aquaculture settings that can lead to severe economic losses. Outbreaks can progress rapidly in aquaculture settings because fish are often kept in confined quarters resulting in physical and
physiologically stressful conditions. Overcrowding and other environmental stresses including poor nutrition, poor water quality, improper handling and the presence of other disease causing agents may increase the chances of infection (Reed and Francis-Floyd, 1996). Parasites that damage fish tissue, as well as wounds acquired during fighting can create ideal locations for secondary infection by *Vibrio* spp. Initially, infections can cause lethargy and loss of appetite and as the disease progresses, boils may appear on the body of fish which can then develop into large, open sores. When disease becomes systemic the gut and rectum of the fish may become bloody and fluid filled. Once an outbreak is in progress, the number of infectious particles in the water rise dramatically, increasing the likelihood of exposed fish becoming infected (Reed and Francis-Floyd, 1996).

Fish diseases caused by *Vibrio* species have been reviewed extensively by many investigators and, among the many fish pathogens, *V. anguillarum* has been recognized historically as a major pathogen of marine fish and other marine animals (Colwell, 2006). Vibriosis caused by *V. anguillarum* has been recognized as a major obstacle for salmonid marine culture where epizootics can lead to a rapid loss of farmed fish. *V. anguillarum* is thought to adhere to skin mucus and invade lesions on the skin of injured fish. In addition, orally ingested cells can adhere to the intestinal tract of fish leading to systemic infection (O’Toole et al., 1999). More recently, Crosa et al. (2006) reported that water temperature, water quality, virulence of the *V. anguillarum* strain, and stress on the fish were important elements influencing the onset of disease outbreaks. Clinical signs of infection include red spots on the ventral and lateral areas of the fish that can ulcerate, as well as corneal lesions. However, in acute and severe epizootics, the course of infection
is rapid, and most infected fish die without showing any clinical signs (Crosa et al., 2006). Several virulence-related factors and genes have been identified including polar flagellar genes, genes involved in the production of metalloproteases, and hemolysis genes (Rodkhum et al., 2006). However, the mechanism of pathogenesis as a whole has not been fully understood.

A second *Vibrio* sp. implicated in opportunistic infections in marine fish is *V. ordalli*. This species is responsible for severe economic losses in the culture of several salmonid species in the coastal waters of America’s Pacific Northwest, Japan, Australia and New Zealand (Fernandez and Avendano-Herrera, 2009). Unlike infections caused by *V. anguillarum*, *V. ordalii* infections are localized in the muscle and skin and can cause necrosis and hemorrhaging of the surrounding tissue. Infections generally occur as a result of the invasion of the skin of fish, however *V. ordalii* has also been isolated from loose connective tissue, gills and throughout the digestive tract, suggesting that infection could also begin at these sites. Correlation between the presence of *V. ordalii* in the blood of fish and the decrease in white blood cell (leukocyte) counts in dying fish suggests that the production of a leukocytolytic factor may play an important role in the pathogenesis of the infection (Crosa et al., 2006).

Other species of *Vibrio* capable of causing infection in marine fish include *V. salmonicida*, *V. splendidus*, *V. ichthyoenteri* and *V. damsella*. These species cause a variety of clinical manifestations in fish, mollusks, eel and crustaceans (Austin, 2006) including abdominal hemorrhaging and intestinal necrosis. The Food and Agriculture Organization of the United Nations estimates that the world’s seafood demand will be met by aquaculture in 2020 as wild capture facilities are overexploited and are in decline.
Bacterial infections, particularly by species of *Vibrio*, can have a major impact on fish, shrimp and shellfish grown in aquaculture settings. This can lead to major economic losses to what is more than a $1 billion industry in the U.S. alone (USDA, 2005).

1.5. OVERVIEW OF *VIBRIO* SPP. AS PATHOGENS OF HUMANS

*Vibrio* spp. are known to cause opportunistic infections in humans. Table 1.1 includes the 12 species that are known to infect humans and are capable of causing mild to severe gastroenteritis, wound infections, septicemia and death. The majority of human infections are attributed to four species of *Vibrio*: *V. cholerae*, *V. vulnificus*, *V. parahaemolyticus* and *V. alginolyticus*. According to the Centers for Disease Control (CDC), between 2007 and 2009, there were more than 1,800 cases of human infections attributed to *Vibrio* species (MMWR, 2010). It is thought that these documented cases are only a fraction of actual infections because of underreporting. Most infections go unreported because they can be self-limiting and do not require medical attention. In addition, many clinicians may not recognize infections by these bacteria, which may result in many cases going undiagnosed. The majority of illness in the U.S. occurs in the Gulf Coast region, but, this may be due to the fact that surveillance has been better in this area. Moreover, the warmer waters of the Gulf region are more conducive for growth of *Vibrio* spp. and this area has most of the industry that produce the seafood (oysters, clams, shrimps) involved in transmission of *Vibrio* spp. to humans. In 1988, The Cholera and Other *Vibrio* Illness Surveillance (COVIS) system was initiated by the CDC, U.S. Food and Drug Administration (USFDA) and the Gulf Coast states (Alabama, Florida, Louisiana, Mississippi and Texas). This surveillance system serves as a national database.
for reported human illnesses caused by all species of *Vibrio* and the information generated has been used to educate consumers about the health risks associated with these bacteria. Up until relatively recently, reporting of *Vibrio* species related infections was not mandatory in the U.S., however as of 2007, reporting of infections by all species of *Vibrio* to the CDC has become mandatory. Thus, we can expect that numbers related to infections by these bacteria will increase as a result of better surveillance.

It should be noted that *Vibrio* spp. are largely opportunistic pathogens. As a result, environmental conditions, and the relative susceptibility of the host organism to these bacteria play major roles in the likelihood of infection and in the severity of disease symptoms. In this regard people with underlying medical conditions that result in “immunocompromised systems” are known to be highly susceptible to infection by *Vibrio* spp. and symptoms leading to death. Another well known compromising situation are preexisting wounds on the skin surface that allow *Vibrio* spp. to gain entrance and initiate an infection.

1.6. HUMAN PATHOGENIC *V. CHOLERAE*

1.6.1. The Disease: Pandemic Cholera Was a Historical Scourge of Mankind

*V. cholerae* is the cause of the disease called cholera, which is listed as one of the greatest scourges to mankind. This disease has killed a large number of people throughout the recorded history of people in every major continent (Rodrique et al., 1994). Cholera has been endemic in India for many years and became pandemic when populations became mobile and armies began invading India and many other countries. There have been seven pandemics of cholera over the recorded history of mankind (Kaper et al., 1995). The first recorded pandemic of cholera (1817-1823) started in
Calcutta, India and greatly affected the population in India and its neighboring countries, including China, Japan, Philippines as well as the Middle East and Russia. The first pandemic signified the first time cholera had moved out of south Asia, beginning a series of pandemics that dominated the world’s disease history in the 19th century. The spread of cholera was facilitated by western military and colonial power and rapid improvement in the speed and frequency of world traffic (Hays, 2005). The second pandemic (1829-1851) started in the Indian continent and reached the British Isle in the early 1830’s. By 1832 cholera had devastated the populations of London and Paris and reached the New World (Canada and U.S.) via ships from Ireland carrying immigrants who sustained cases of cholera during the Atlantic crossing. The disease spread from Montreal to New York,

![Timeline of the eight cholera pandemics throughout history.](image)

Philadelphia, Baltimore and Washington, D.C. The third pandemic of cholera (1852-1859) spread through most of Europe, the Middle East, North Africa as well as the west coast of the U.S. where it was carried by wagon trains of pioneers. During the fourth pandemic, which lasted from 1863 to 1879, cholera continued to spread throughout the U.S. reaching Louisiana, Mississippi, Missouri and Ohio by the early 1870s. The fifth cholera pandemic extensively affected South America, causing large epidemics.
accompanied by high mortality in Argentina, Chile and Peru (Kaper et al., 1995), and reappeared in Russia where it is estimated that 215,000 Russians died as a result of this disease (Hays, 2005). The 6th cholera pandemic involved populations in the Near and Middle East, and remained virtually confined to south and southeast Asia from the mid-1920s until the onset of the 7th pandemic in 1961 (Kaper et al., 1995). This pandemic of cholera is currently ongoing, and unlike previous pandemics, which had originated in the Indian subcontinent, originated from Indonesia. In addition, the causative agent of this pandemic is \( V.\ cholerae \) O1 biotype El Tor while the previous two pandemics (5th and 6th) were attributed to the classical biotype of this serogroup. The 7th pandemic has been the most extensive in terms of geographical spread and time. \( V.\ cholerae \) O1 El Tor spread from Indonesia to the Philippines and Taiwan affecting nearly the entire southeast Asian archipelago. In the 1970’s outbreaks of cholera began in the Middle East and West Africa which resulted in more than 400,000 cases with a high case fatality (Kaper et al., 1995). By 1991 cholera was once again seen in South America beginning with epidemics in Peru and then spreading to south and central America. This represented the first time cholera had entered this continent in more than a century. By late 1992 epidemic cholera was seen in Madras, India and Bangladesh. The causative agent was not \( V.\ cholerae \) O1 as with previous epidemics but rather \( V.\ cholerae \) expressing a distinct surface antigen (O139). This serogroup has since spread throughout India, Pakistan, China and Thailand and imported cases have also been reported in the U.S. and United Kingdom (Kaper et al., 1995).

Up through the third cholera pandemic, the cause of the disease was not known. Most people at that time believed in the “Miasma Theory,” or that diseases were caused
by poisonous vapors or miasmas. It was believed that that these vapors, characterized by a foul smell, were carriers of particles of decaying matter. At this time industrialization and urbanization had led to dirty, foul smelling city neighborhoods which were the epicenters of disease and epidemics, while improvements in sanitations and cleanliness of these areas led to a decrease in disease. These two observations gave credibility to the miasma theory during this time. It was not until the mid-1800’s that the Germ Theory of disease, or the theory that microorganisms were the cause of disease, began to gain acceptance. The first direct demonstration of the role of bacteria in causing disease, however, did not occur until 1880 when *Bacillus anthracis* was discovered by Robert Koch (Schneerson et al., 2003).

Two major scientific studies in relation to cholera were made around 1854. In London, John Snow conducted the first epidemiological study which showed that drinking water contaminated by human sewage was the cause of cholera disease. Snow mapped the households with cholera and was able to trace the cases to a sewage contaminated water pump on Broad Street. About the same time Filippo Pacini, a medical student in Italy, performed autopsies on deceased cholera patients during an epidemic in Florence. Using a microscope he discovered a bacillus, which he described as a “vibrio” with a comma-like shape, from the intestinal mucosa of people who had died from cholera. Pacini published his findings of the destruction of the intestinal mucosa during cholera and stated that this vibrio was the specific causative agent of the disease. Pacini’s data was largely ignored by the scientific community, and the etiological agent of cholera was rediscovered by Robert Koch in 1884, one year after Pacini’s death (Bentivoglio and Pacini, 1995). Koch, unaware of the findings of Pacini,
independently identified the “bent shaped bacillus” as the cause of cholera. Koch was able to grow the cholera bacilli in pure culture, but he could not fulfill his own Koch’s postulates because the animals he used were not susceptible to cholera disease. Though the credit of the discovery of \textit{V. cholerae} generally goes to Koch, the International Institute of Microbiology in 1965 voted to recognize the contribution of Pacini by renaming \textit{Bacillus cholerae} as \textit{Vibrio cholerae} (Colwell, 2006).

1.6.2. Current Assessment of Cholera in the World and in the United States

Historically, cholera served as the model disease that initially caused fear and havoc throughout the history of mankind, but was later conquered by developments in science. The disease symptoms of cholera are easily recognizable because it causes the infected to rapidly lose body fluids through watery stools, resulting in agonizing suffering and death within a few hours of the onset of symptoms. Cholera has caused great fear among people and its impact changed the course of history as many soldiers and leaders were killed. Though both John Snow and Filippo Pacini completed their careful studies during the third pandemic of cholera, it was not until the fifth pandemic and Robert Koch’s work that it was accepted that a bacterium was the cause of cholera.

Currently, cholera occurs in developing counties (e.g., India, Pakistan) with poor sanitation and inadequate environmental laws. Worldwide, cholera cases can number in the hundreds of thousands. In 2005, for example, there were more than 130,000 reported cases of cholera worldwide, 125,000 of which were from the continent of Africa (WHO, 2006). The actual number of cases, however, is thought to be much higher due to underreporting. According to the World Health Organization (WHO, 2010) about 20% of individuals infected with cholera develop acute watery diarrhea, of which 10-20%
develop severe water diarrhea with vomiting. If left untreated, the case fatality rate for cholera can be 30-50%, but with treatment fatality rates are less than 1% (WHO, 2010). Outbreaks of cholera can also cause panic and can disrupt the social and economic structure of a country or area. Panic induced reactions by other countries include curtailing or restricting travel from countries where a cholera outbreak is occurring, or import restrictions on certain foods. For example, the 1991 cholera outbreak in Peru cost the country US$ 770 million due to food trade embargoes and adverse effects on tourism (WHO 2010). In developed countries with good sanitation and environmental laws cholera is nearly absent. According to the CDC cases of cholera that occur in the U.S. are acquired from international travel or from the consumption of contaminated seafood from the Gulf Coast or foreign waters.

Outbreaks of cholera occur generally as a result of deteriorating water quality and sanitation standards which often results as an aftermath of natural disasters. An outbreak in Papua New Guinea, beginning in August of 2009, has so far affected more than 1,200 people in that country. This outbreak was the first time cholera had been seen in Papua New Guinea in 50 years, which has lead to challenges in raising awareness about treatment, prevention and the stigma associated with the disease. Though the death toll has been low, poor hygiene and water quality as well as poor sanitation standards have been associated with the yearlong spread of cholera throughout the country. In July 2010 heavy monsoon rains in Pakistan triggered the worst flooding in the history of the country. It was feared that outbreaks of cholera would soon follow the flooding, however only one confirmed case of the disease has been seen. Unlike in Papua New Guinea the WHO’s warning system to detect cases of cholera, and other waterborne diseases
associated with flooding, has attributed to the low death toll due to cholera in Pakistan.

With the help of international and Pakistani aid workers, clinics were setup to deal with cases of waterborne disease and helicopters were available to quickly transport those suffering from diarrheal disease to these centers. In addition, radio and text message networks were setup to disseminate information to the public about the importance of good hygiene and washing hands. These type of control measures may have had a significant impact on the spread of cholera and contributed to the low death toll in Pakistan where officials were expecting high incidence of disease and death. In central Africa, where cholera is endemic, an unusually high number of cases (>40,000) and deaths (>1,500) of this disease has been seen in Cameroon, Chad, Niger and Nigeria in the last few months. Flooding, poor hygienic conditions and population movements have contributed to the high number of cases in these four countries. WHO is working on strengthening surveillance in these areas and providing supplies (chlorine to disinfect water) for management of cases.

Of significance is an outbreak of cholera that is ongoing in Haiti, which is located in the western hemisphere and where cholera has not been previously detected. The source for the importations of \textit{V. cholerae} is not known but there is some evidence that Nepalese soldiers sent to Haiti for peace keeping duties may have imported the disease to Haiti since cholera is endemic in Nepal. The epidemic is ongoing with more than 12,000 cases and over 1,000 deaths reported. Spread of this disease occurred as an aftermath to a powerful earthquake that devastated the country in January 2010. The earthquake resulted in a crippled infrastructure and the displacement of Haitians into temporary
settlements. The disruption of water and sanitation systems, along with overcrowding at emergency settlements, created ideal conditions for the epidemic spread of cholera.

1.6.3. Cholera Infections in Hawaii

Asiatic cholera arrived in Hawaii on the ship Belgic, which arrived in Honolulu from Yokohama in August 1895. More than 500 Chinese immigrants were aboard, a handful of whom died en route and others who were found sick on arrival (Forbes, 2003). Since the cases in 1895, no new cases of cholera were documented in Hawaii until 1991 when two confirmed cases of cholera were found in Honolulu. Both individuals lived on Oahu and had not travelled outside of the state. Consumption of contaminated raw fish was thought to be the likely source of infection, however site visits to fish distributors failed to recover *V. cholerae* (Mintz et al., 1994). In 2005 two more cases of cholera linked to seafood, from two different restaurants, were seen in Honolulu (Leidemann, 2005). Both individuals suffered abdominal problems but did recover.

1.6.4. Transmission, Pathogenesis, Environmental Factors and Virulence Markers

Cholera infections are generally acquired from drinking water that has been contaminated by the feces of a symptomatically or symptomatically infected person or through contaminated seafood. The dose needed to establish infection in healthy individuals is $10^6$ to $10^{11}$ CFU (Prouty and Klose, 2006) but may be lower in individuals who take antacids. This is because antacids decrease the acidity of the stomach which facilitates passage of *V. cholerae* cells through the digestive tract. Very few cases of cholera occur in industrialized nations due to improvements in sanitation, however, it remains endemic in Africa and South and Southeast Asia (Mintz, 2009). A handful of
cases of cholera do occur in the U.S. and are attributed to ingestion of contaminated
seafood or to international travel.

Today over 200 serogroups of *V. cholerae* are known to exist, but the eight
pandemics of cholera that have occurred have been attributed to only two serogroups of
*V. cholerae*, O1 and O139. Only toxigenic strains (those that contain the cholera toxin
gene) of serogroups of O1 and O139 have caused widespread epidemics and are
reportable to the WHO as “cholera.” Other serogroups of *V. cholerae*, i.e., those that do
not contain the cholera toxin gene (non-toxigenic strains) including non-toxigenic O1 and
O139 strains, are capable of causing cholera like illness. *V. cholerae* O1 has two
biotypes, Classical and El Tor, and each biotype has two distinct serotypes, Inaba and
Ogawa. Symptoms between biotypes are indistinguishable though a higher proportion of
persons infected with the El Tor biotype remain asymptomatic or only develop a mild
illness (Mintz, 2009).

The initial stage of *V. cholerae* pathogenesis involves the bacteria reaching the
upper intestinal tract and attaching to and penetrating cells of the intestines. In humans,
there are generally very few indigenous bacteria in the upper intestinal tract due to the
fact that they must overcome many host barriers. These include surviving the high acid
pH of the stomach followed by the alkaline pH and high levels of digestive enzymes of
the duodenum (Wachsmuth et al., 1994). These harsh conditions may be one reason why
a high infectious dose of *V. cholerae* is needed to initiate infection. The lipopolysaccharide
(LPS) of the microorganism serves as a protective barrier from penetration by bile salts
and other toxic molecules from the gastrointestinal tract. It also slows down the
destruction of the bacterial cell by serum components and phagocytic cells. Once *V.*
*V. cholerae* cells reach the intestines they must deal with a mucous layer of glycoprotein. *V. cholerae* is able to overcome this barrier by secreting proteases that degrade this mucus layer (Silva et al., 2003). Once *V. cholerae* has penetrated the mucous layers it must adhere to the surface of epithelial cells. Bacteriophage VPIΦ encodes determinants essential for *V. cholerae* identification and colonization of proper host environments. Once such determinant is the toxin-coregulated pilus (TCP). TCP is a type IV bundle forming pilus and is the major colonization factor in pathogenic *V. cholerae*. Adherence of *V. cholerae* cells to the intestinal mucosa is mediated by TCP whose gene expression is co-regulated with the expression of cholera toxin genes. Though this pilus has shown to mediate intestinal microcolony formation, the mechanism by which TCP mediates colonization and whether it is the only factor required for colonization is still not known. In addition to its role in the colonization of the host, TCP also plays a major role in virulence by serving as the receptor for the lysogenic, filamentous bacteriophage CTXΦ. Once *V. cholerae* colonizes the upper intestinal tract it secretes its major virulence factor, cholera toxin (CT). The pathogenesis of cholera is due almost solely to the action of CT, an A-B toxin complex whose gene products are encoded on the genome of CTXΦ. CT is a heterodimeric protein made up of an active A subunit and a receptor binding B subunit, and is responsible for severe disruption of intestinal cell function, leading to the watery, secretory diarrhea characteristic of cholera. Within the cells, the toxin increases intracellular cAMP levels which lead to the opening of ion channels and the secretion of chloride ions. This leads to an osmotic imbalance within the cell and causes large amounts of water to flow into the lumen of the intestines, resulting in diarrhea and
dehydration. Over a course of three days, up to 90 liters of diarrhea may be produced (Prouty and Klose, 2006).

Prevention, preparedness and response, along with an efficient surveillance system is key to lessening cholera outbreaks, controlling cholera in endemic areas and in reducing deaths (WHO 2010). Cholera outbreak prevention mainly involves providing access to clean water and proper sanitation in areas where these basic needs are lacking. Education of communities about basic hygienic behaviors, including hand washing after defecation and before handling food or eating, is also important in preventing the spread of infections. The main tools for cholera control include: proper and timely case management in cholera treatment centers, specific training for proper case management, sufficient pre-positioned medical supplies for case management (e.g. diarrheal disease kits), improved access to water and sanitation, enhanced hygiene and food safety practices and improved communication and public information (WHO 2010).

Cholera infections are generally asymptomatic or result in mild gastroenteritis, however severe cases are characterized by vomiting, profuse, watery diarrhea that can lead to dehydration. Illness is easily treatable and up to 80% of people can be treated successfully with prompt oral rehydration salts to replace lost fluids. Intravenous fluids may be required for severely dehydrated patients. These patients also may require antibiotics to diminish the duration of diarrhea, reduce the volume of rehydration fluids needed and shorten the duration of V. cholerae secretion (WHO 2010). The choice of antimicrobial drug used in treatment of cholera is dependent on the patterns of resistance of the infecting strain. Drug resistance in V. cholerae O1 and O139 strains has reduced the number of drugs that have historically been used in the treatment of cholera (Garg et
al., 2000; Lindenbaum et al., 1967). Tetracycline and its derivatives have been used for over 40 years and are effective when used in a single dose (Saha et al., 2006). However, toxicity in certain segments of the population (children, pregnant women) as well as increased drug resistance has limited its use. An alternative, erythromycin, has also been used, however 12 doses over a three day period rather a single dose is required with this drug. Azithromycin, a derivative of erythromycin, has shown potential in the treatment of cholera. However unlike it’s derivative, this drug shows less gastrointestinal toxicity and is effective when used as a single dose for treatment in children (Khan et al., 2002).

1.7. HUMAN PATHOGENIC V. VULNIFICUS

1.7.1 The Disease: A Recent, Fearsome and Deadly Infection of Man

*Vibrio vulnificus* is found in temperate and tropical waters around the world and can be isolated from seawater, sediment, and various marine life forms. *V. vulnificus* was first identified as a new species in 1976, and is considered one of the most virulent human pathogens that can be naturally found in estuarine and coastal waters. It can also be found in high numbers in bivalve mollusks such as oysters and clams (Oliver, 2006). Due to the filter feeding nature of molluscan shellfish, *V. vulnificus* numbers in shellfish tissues are many times higher than levels in surrounding seawater (Tamplin, 2001).

Historically, *V. vulnificus* strains have been classified by biotyping, a technique based on the combination of different phenotypic, serologic, and host range characteristics (Drake et al., 2007). Currently this organism has been classified into three biotypes: biotype 1, biotype 2 and biotype 3. Whereas each is known to be pathogenic to humans, biotype 1 is almost exclusively associated with human disease, and is the biotype of greatest public concern (Oliver, 2006). Biotype 1 causes opportunistic infections in humans and is
responsible for three types of clinical illness in humans: gastroenteritis, wounds infections and primary septicemia. Although gastroenteritis is self-limiting and rarely reported, wound infections and primary septicemia are highly lethal conditions that occur most often among persons with liver disease or other immunocompromised conditions (Gulig et al., 2005). Biotype 2 strains of *V. vulnificus* were initially thought to be only associated with infection in eels, however it is now known that this biotype can also be pathogenic to humans. In 1996, a third biotype of *V. vulnificus* was discovered in an outbreak involving 62 Israeli patients with either wound infections or septicemia (Drake et al., 2007). This biotype has so far not been associated with food consumption.

1.7.2. Current Assessment of *Vibrio vulnificus* Infections Worldwide and in the United States

Human illness due to *V. vulnificus* infections have been reported worldwide, from the U.S. to Europe and Australia (Strom and Paranjpye, 2000). The highest environmental levels of *V. vulnificus* occur in warm months of the year, and these levels parallel the incidence of human infections due to seawater exposure (Tamplin, 2001). Most epidemiological data about this species in the U.S. comes from the Gulf Coast region due to their large shellfish industry and effective surveillance system. Surveillance data shows that most infections occur during the warm weather months and include both infections from eating contaminated seafood as well as illness acquired from wound infections. *V. vulnificus* can cause three types of infections in humans: gastroenteritis, wound infections and primary septicemia. Between 1988 and 1996, there were 422 cases of *V. vulnificus* infection reported in the U.S. Of these, 45% were wound infections, 43% primary septicemia, 5% gastroenteritis, and the remaining 7% from
undetermined exposure (Shapiro et al. 1998). All cases of primary septicemia were a result of the consumption of raw oysters and clams, while contact with waters or shellfish were the cause of *V. vulnificus* wound infections. Wound infections were either a result of infection of a preexisting wound or a wound sustained at the time of exposure. Many wounds were related to occupational exposures among oyster shuckers and commercial fisherman (Strom and Paranjpya, 2000). Gastrointestinal infection due to *V. vulnificus* is thought to be largely underreported due to the fact these infections are generally mild, require no medical treatment, and are not life threatening. However it is difficult to definitively state that *V. vulnificus* can cause seafood-related gastroenteritis as most clinical isolations of *V. vulnificus* have not been accompanied by screening for other potential causes of illness, including viruses or other non-vibrio bacterial pathogens (Strom and Paranjpya, 2000).

*V. vulnificus* is the number one cause of seafood-borne death in the U.S. (95% of all seafood related deaths), with about 50 cases serious enough to require hospitalization each year (Oliver 2006). In addition, *V. vulnificus* has a high fatality rate of 50-60% due to the consumption of raw or undercooked seafood. Japan may have one of the highest rates of infection because of its warmer coastal waters, which are conducive to *V. vulnificus* growth, and because of higher levels of raw seafood consumption (Gulig et al., 2005). Thus, gastroenteritis acquired through the consumption of raw or undercooked seafood can present a large public health risk to humans. However, gastroenteritis related *V. vulnificus* infections are the least significant of infections because they are relatively mild and are largely not reported. Symptoms include diarrhea and stomach cramps, and infections rarely require antibiotic intervention. No fatalities from *V. vulnificus*
gastroenteritis have been reported (Oliver, 2006). Unlike with cholera, \textit{V. vulnificus}
infections are not associated with epidemics. However, severe infections leading to
bloating and amputation of limbs, along with rapid onset of death cause panic and fear
among populations. \textit{V. vulnificus} is one of the pathogenic bacteria that cause necrotizing
fasciitis or flesh eating bacteria that often result in death. Although \textit{V. vulnificus}
infections are relatively rare this pathogen has had a profound effect on public health
policies, including those in Florida, California, and Louisiana, where consumer advisories
must be posted at points of retail sale for raw molluscan shellfish (Tamplin, 2001).

\textbf{1.7.3. \textit{Vibrio vulnificus} Infections in Hawaii}

There have been several documented cases of \textit{V. vulnificus} infection in Hawaii.
All infections have been wound infections associated with contact with recreational
waters. Prior to 2007, the reporting of infections by this microorganism to the CDC was
not mandatory, thus, many cases may have gone unreported. In 2001, a 72 year old
visitor from California contracted a \textit{V. vulnificus} infection and died two days after
swimming in Ahalanui Hot Pond on the Island of Hawaii. Water temperature in this
thermal pond, which is heated by underground lava, is known to be very warm (34°C)
and is conducive to the growth of this bacterium. In addition, this individual was known
to have psoriasis and while swimming in the pond was bitten by fish. Upon his death his
wife successfully sued the State of Hawaii requiring a sign to be placed at the pond
warning swimmers of the risk of contracting wound infections. In 2002 a 77 year old
individual who swam at the same pond became ill but recovered. A more recent death
related to \textit{V. vulnificus} occurred in 2006 when a 34 year old individual fell into Ala Wai
Canal. In the days prior to him falling into the canal, a large sewage spill had occurred in
the Waikiki area forcing 48 millions of untreated sewage to be diverted into the canal.
The individual had chronic liver disease and cuts and abrasions on his body at the time of
his fall into the canal, and he succumbed to his infection six days later. This death served
to bring public awareness to infections caused by *V. vulnificus* and other types of *Vibrio*
species.

1.7.4. Transmission, Pathogenesis, Environmental Factors and Virulence Markers

In addition to gastrointestinal infections associated with the consumption of
contaminated seafood, *V. vulnificus* can also cause life threatening wound infections in
people who recreate in waters where they are found. These infections can be either to
preexisting wounds or wounds acquired during handling of seafood (i.e. shucking
oysters). The wound does not have to be significant; several cases of persons who
suffered ant bites on their legs or hands before or after contact with coastal waters
developed wound infections which were either fatal or resulted in limb amputation
(Oliver, 2006). The incubation period can range from three hours to twelve days but
most symptoms begin within 24 hours of infection and death can occur within hours of
hospitalization (Torres et al., 2002). Symptoms of *V. vulnificus* wound infection include
pain, redness and swelling at the wound site. The cellulitis may proceed to deeper tissue
and cause extensive damage (necrotizing fasciitis) to skeletal muscle. Wound infections
that become necrotic may require amputation, and, in certain individuals, may develop
into a life threatening secondary septicemia (Oliver, 2006). No predisposing conditions
are necessary for individuals to develop wound infections, however those who develop
secondary septicemia generally have problems related to their liver. A primary
predisposing host condition is high levels of tissue iron, which is classified as
hemochromatosis, a result of gene mutations and liver disease, such as cirrhosis from alcoholism or hepatitis (Tamplin, 2001). These individuals tend to have elevated levels of serum iron, which has been experimentally shown to enhance *V. vulnificus* growth (Wright et al, 1981). Iron is an important limiting growth factor for these bacteria, and elevated levels of this compound can lead to rapid growth of this species.

Primary septicemia is the most significant form of *V. vulnificus* infection, and generally results from the consumption of contaminated seafood. Generally infections occur during the warm water months of May through October when *V. vulnificus* cells are present in seawater and shellfish (Oliver, 2006). Sepsis generally develops in persons with underlying medical conditions and is rarely seen in healthy individuals. Of the infections occurring in the U.S. between 2000 and 2003, 94% were reported to have one or more underlying diseases, the most common of these were alcoholism or alcohol abuse, or infections such as hepatitis, which generally leads to liver damage, including cirrhosis (Oliver, 2006). Diabetes, low stomach acidity (due to antacid use), cancer and HIV infection have also been implicated as risk factors for the development of disease (Oliver, 2006). In addition, most cases of primary septicemia occur in males over the age of 50. This may be attributed to the fact that men are more than twice as likely to eat raw oysters as women, the large number of men in the population with liver disease and alcoholism and the larger percentage of men with occupational exposure to raw seafood drippings and seawater (Shapiro et al., 1998). Symptoms of primary septicemia generally develop within 36 hours and include fever, nausea and hypotension. A common and highly characteristic symptom associated with septicemia is the development of secondary skin lesions. These lesions begin as fluid filled blisters, typically on the legs.
and feet, and result in tissue and muscle destruction (necrotizing fasciitis) (Oliver 2006). Removal of damaged tissue or amputation is generally required to treat patients at this stage of infection. Infections develop rapidly and death can occur within days of hospitalization. Both primary septicemia and wound infections are noted for the extremely rapid replication of bacteria in host tissue with extensive tissue damage to the skin. Even with treatment, case fatality rates (CFRs) for septicemia can be as high as 75% and CFRs for wound infection can be as high as 50% (Hlady and Klontz., 1996). Additionally, death can occur within 24 hours after contact with *V. vulnificus*.

Symptoms of primary septicemia include the sudden onset of chills and fever, accompanied by vomiting, diarrhea and abdominal pain. Secondary lesions may also develop on the extremities of these patients which often become necrotic and require amputation. The CFR for individuals with this type of *V. vulnificus* infection can be greater than 60%. Individuals with wound infections generally have pain and inflammation at the wound site, and wounds can become necrotic as with primary septicemia. Wound infections can become septicemic but CFRs (20-30% of infections) are lower than those seen with primary septicemia. Gastrointestinal symptoms include fever, diarrhea, abdominal cramps and vomiting. Infections are generally self limiting and tend to go unreported (Strom and Paranjpya, 2000).

The infectious dose of *V. vulnificus* is unknown; however it is known that individuals who have liver disease (hepatitis, cirrhosis) are more susceptible to infection. Other chronic conditions such as diabetes mellitus, chronic intestinal disease or low gastric acid are also risk factors for infection (Koenig et al., 1991; Shapiro et al., 1998). In a Gulf Coast survey, preexisting conditions played the biggest role among patients
with primary septicemia; 97% of these patients had preexisting conditions. In those with wound infections, 68% had predisposing conditions while these factors were only present in 35% of individuals with gastroenteritis. Furthermore, liver disease was a strong predictor for fatal outcomes of \textit{V. vulnificus} infection as 80% of fatalities had liver disease as opposed to 35% of nonfatal infections who had liver disease (Shapiro et al., 1998). Despite the fact that a link may be seen between liver disease and \textit{V. vulnificus} infection and death, infections due this bacteria are generally rare. Thus, unknown factors of both the host and the pathogen may be involved in the disease process.

The two main virulence factors associated with \textit{V. vulnificus} are its capsule and LPS. The presence of a polysaccharide capsule is the most documented virulence factor. Only capsulated strains are capable of initiating infection in humans due to the capsule’s anti-phagocytic properties. Animal studies have shown that the dose fatal to 50% (LD$_{50}$) of mice injected with capsulated and non-capsulated strains showed a remarkable difference. LD$_{50}$ values for non-capsulated strains were generally $>10^8$ cells, whereas LD$_{50}$ values for capsulated strains were less than 10 cells (Wright et al., 1981). Under laboratory conditions, cells can switch from capsulated to non-capsulated forms at a frequency between 0.01% and 1% depending on the strain and growth conditions, however reversion from the non-capsulated to the capsulated form have not been documented (Oliver, 2005). More than 10 capsular serotypes have been reported but it is not known whether one serotype is capable of initiating illness over another (Wright et al., 1999).

The LPS is a second virulence factor associated with \textit{V. vulnificus}. It is responsible for the shock and death associated with infection. Symptoms which occur
with *V. vulnificus* septicemia, as well as the inflammatory response observed in wound infections, are those associated with the endotoxic activity of LPS molecules (Linkous and Oliver, 1999). Laboratory studies have shown that the female hormone estrogen may play a protective role against LPS toxicity. When male and female mice were injected with LPS extracts at a dosage fatal to the majority of male mice, 82% of male mice died as compared to only 21% of female mice. When estrogen levels in female mice were depleted their fatality rate increased to 75%. Thus, the host response to *V. vulnificus* is significantly different for males and females, and while the exact mechanism of this response difference is not understood, it may explain why males who consume raw oysters make up the major risk group for *V. vulnificus* primary septicemia (Linkous and Oliver, 1999).

Putative virulence factors such as cytolysin/hemolysin and a metalloprotease have also been identified in *V. vulnificus*. These two factors were believed to be important for *V. vulnificus* infection. However, some studies (Fan et al., 2001; Wright and Morris, 1991) have shown that they may not play an essential role in the development of primary septicemia, but rather play a role in wound infections. In addition, flagella and a recently identified toxin called RTX have been identified as potential virulence factors. However, more studies need to be done in order to elucidate the role they play in the pathogenesis of *V. vulnificus*.

Based on laboratory studies, virtually all *V. vulnificus* strains are virulent, however only a few cases of wound and primary septicemia occur in the U.S. each year. For example, only 30 or fewer fatal cases are typically reported in the U.S. each year, despite USFDA estimates that 20 million people consume 75-80 million servings of raw
oysters annually, and that 12-30 million persons have one or more known risk factors for *V. vulnificus* infection (Oliver, 2006). Thus, it is likely that there may be other factors required to predispose a person to infection and that laboratory studies based on mouse models are inconclusive. All “clinical” strains have had to derive from environmental strains, and the virulence factors a strain must possess to allow it to become infectious are not fully understood, thus, genetic variation among environmental *V. vulnificus* strains may provide a better understanding about its pathogenesis (Oliver, 2006).

Successful treatment of *V. vulnificus* infections is highly dependent on receiving prompt medical treatment, however there are currently no well-established management protocols (Kuo et al., 2007). Prompt antimicrobial therapy is essential for the treatment of infection, and should be initiated as soon as a diagnosis is made. Antimicrobial therapy with a combination of agents, such as cefotaxime and minocycline, have shown better outcomes that monotherapy (Kuo et al., 2007). In patients presenting symptoms of necrotizing fasciitis, surgical debridgement, incision and drainage of abscesses and sometimes amputation have been shown to reduce mortality and shorten hospitalization (Bross et al., 2007). Prevention of infection is the key when dealing with *V. vulnificus* infection. Individuals who are immunocompromised or have chronic liver disease should not eat raw seafood, and should avoid swimming in warm waters where these microorganisms inhabit.
1.8. HUMAN PATHOGENIC V. PARAHAEOMOLYTICUS

1.8.1. The Disease: Historically a Seafood Borne Diarrhea

*V. parahaemolyticus* was first isolated and described in the 1950’s in Japan, and has since been the subject of extensive study as the causative agent of numerous seafood-borne outbreaks in Japan and other Asian countries (Tamplin, 2001). This organism can be isolated from a variety of marine environments including seawater, sediment, shellfish and plankton, and is less restricted to low salinity, warm waters as *V. cholerae* and *V. vulnificus*. *V. parahaemolyticus* is an enteric pathogen that causes acute gastroenteritis in humans, generally as a result of consumption of raw or undercooked seafood (DePaola et al., 1990). It has been recognized as a worldwide cause of food-borne gastroenteritis, particularly in the Far East, where seafood consumption is high (Nair et al., 2007).

*V. parahaemolyticus* can cause three types of clinical disease: gastroenteritis, wound infections and septicemia. The most common type of infection is gastroenteritis and symptoms include diarrhea, abdominal pain, fever, nausea and vomiting. Diarrhea is generally watery, mucoid and bloody but infections are usually self-limiting (Iida et al., 2006). The mean incubation period of *V. parahaemolyticus* infection is 15 hours and infections can generally last an average of three days in patients with no underlying conditions (Nair et al., 2007). Though less common, *V. parahaemolyticus* can also cause an infection of the skin when an open wound is exposed to warm seawater. Wound infections are usually mild infections and rarely become necrotic (only seven cases have been previously reported) (Tena et al., 2010).
1.8.2. Current Assessment of *Vibrio parahaemolyticus* Infections in the World and in the United States

*V. parahaemolyticus* has been the most common *Vibrio* species isolated from humans in the U.S. Between 1988 and 1997, a total of 345 cases of *V. parahaemolyticus* infection were reported to the CDC by states participating in the Gulf Coast *Vibrio* Surveillance System. Of the 345 patients whose cases were reported to the CDC, 202 (59%) had gastroenteritis, 118 (34%) had wound infections, and 17 (5%) had septicemia (Daniels et al., 2000). Most infections of *V. parahaemolyticus* result in gastroenteritis due to the consumption of raw or undercooked shellfish. Symptoms include watery diarrhea, abdominal cramps, nausea and vomiting. Onset of illness generally begins 4 to 96 hours after the consumption of contaminated seafood. Generally gastroenteritis is self-limiting and can last between 2-10 days. *V. parahaemolyticus* can also cause wound infections and septicemia. Infections can be severe in individuals with underlying conditions such as leukemia, liver disease and diabetes (Faruque and Nair, 2006). *V. parahaemolyticus* infections can have fatality rates greater than 50% if left untreated, however the fatality drops to less than 1% in patients receiving treatment.

*V. parahaemolyticus* is recognized as a pathogen in both developed and developing countries. It is an important etiological agent of diarrhea in Calcutta, India, where gastroenteritis ranks second to cholera (Faruque and Nair, 2006). A relatively new serotype of *V. parahaemolyticus*, O3:K6, was discovered to be the cause of a large number of cases of diarrhea in this region. This serotype was also implicated in cases of food poisoning in Japan beginning in 1996 replacing the previous serotype (04:K8) which was the predominant serotype in previous outbreaks (WHO, 1999). Prior to this, there was little evidence that specific serotypes were selectively spreading in geographical
regions (Tamplin, 2001). *V. parahaemolyticus* is now endemic in more than eight
countries, and the emergence of additional pandemic serotypes have been documented
(Chowdhury et al., 2000). Thus, this organism is an emerging pathogen that has become
endemic in many regions of the world.

### 1.8.3. *Vibrio parahaemolyticus* Infections in Hawaii

Several outbreaks due to *V. parahaemolyticus* have been documented in Hawaii.
A significant outbreak of *V. parahaemolyticus* gastroenteritis occurred in 1972 in Hawaii
(Barker, 1974) where thirty-one individuals suffered diarrhea after ingesting raw crab.
No fatalities were reported. Between July and August 1997 the largest reported outbreak
in North America of culture-confirmed *V. parahaemolyticus* infections occurred (CDC
1998). Several states, including Hawaii, were part of this outbreak, which was attributed
to consumption of shellfish harvested from Washington or British Columbia (Canada).
The actual number of *V. parahaemolyticus* associated infections may not be known for
several reasons. First, it is likely that the number of cases of *V. parahaemolyticus*
gastroenteritis may be higher in Hawaii because, similar to Japan, the consumption of
raw seafood is high. Second, because illness is generally self limiting individuals are not
as likely to seek medical treatment. Finally, prior to 2007 *V. parahaemolyticus* was not a
notifiable disease, thus many cases may have gone unreported.

### 1.8.4. Transmission, Pathogenesis, Environmental Factors and Virulence Markers

Symptoms of wound infections can begin from hours to days and consist of
swelling, redness and pain. Early diagnosis of necrotizing fasciitis is difficult and the
disease can be mistakenly diagnosed as cellulitis, resulting in delayed management. In
individuals with underlying health problems, particularly liver disease, these bacteria can spread into the blood and cause septicemia. Though some \textit{V. parahaemolyticus} infections may require hospitalization, the disease is rarely fatal. According to the CDC, an estimated 4,500 cases of \textit{V. parahaemolyticus} infections occur each year in the U.S., however the actual number of cases is thought to be higher due to underreporting. The infectious dose of \textit{V. parahaemolyticus} associated gastroenteritis was determined to be between $10^5$ to $10^7$ organisms (Daniels et al., 2000), however the dose required for wound infections is not yet known.

Strains isolated from diarrheal patients are generally hemolytic and capable of lysing red blood cells on Wagatsuma agar while isolates from food are mainly non-hemolytic. The ability of strains of this organism to cause hemolysis on this agar was termed the Kanagawa phenomenon (KP) and was closely associated with gastrointestinal disease (Iida et al., 2006). KP is a type of beta-hemolysis induced by the thermostable direct hemolysin (TDH) on Wagatsuma agar. The hemolysin was named thermostable direct hemolysin (TDH) on the basis of its characteristics: TDH was not inactivated by heating at 100°C for 10 min, and the hemolytic activity was not enhanced by the addition of lecithin, indicating a direct action on erythrocytes (Sakurai et al., 1973). \textit{V. parahaemolyticus} is capable of hemolysis of various species of erythrocytes, cytotoxicity and fluid accumulation. Most (90%) of the strains isolated from clinical cases show this type of hemolysis, while only 1 to 2% of the strains of environmental origin are KP positive (Nishibuchi and Kaper, 1995). A second hemolysin, TDH-related hemolysin (TRH), was identified in strains that were hemolytic but KP negative. TDH and TRH are encoded by the \textit{tdh} and \textit{trh} genes, respectively, and are considered the main virulence
factors of this microorganism (Nishibuchi and Kaper, 1995). \textit{V. parahaemolyticus} strains isolated from diarrheal patients produce either TDH, TRH or both while environmental strains are generally incapable of producing these hemolysins (Nair et al., 2007). Thus, the genes for these two hemolysins have been used as markers to distinguish virulent strains from non-virulent ones.

Though hemolysins are considered the major virulence factor of \textit{V. parahaemolyticus}, other host factors such as iron and bile may contribute to this microorganisms pathogenicity. Regulation of iron in pathogenesis has not been fully characterized but under iron restricted conditions, \textit{V. parahaemolyticus} strains are known to produce siderophores and use heme and hemoglobin as sources of iron. In addition, it has been proposed that the presence of bile in the human intestines may stimulate TDH production. Studies have shown that the addition of bile to estuarine water led to an increase in colony counts of virulent strains of \textit{V. parahaemolyticus} (Tamplin, 2001). The role and degree to which iron and bile play in the pathogenesis of \textit{V. parahaemolyticus} still needs to be elucidated.

Treatment for \textit{V. parahaemolyticus} infections are not necessary in most cases because there is no evidence that antibiotic treatment decreases the severity or length of illness. Patients are recommended to drink plenty of liquids to replace fluids lost through diarrhea, and in severe or prolonged illnesses antibiotics such as tetracycline, ampicillin or ciprofloxacin can be used (Zulkifli et al., 2009). Surgical debridement may be necessary for cases of necrotizing fasciitis.
1.9. HUMAN PATHOGENIC V. ALGINOLYTICUS

1.9.1. The Disease: An Opportunistic Skin Infection

*V. alginolyticus* was originally classified as a biotype of *V. parahaemolyticus* until it was made a distinct species based on sucrose fermentation and other phenotypic properties (Nishibuchi, 2006). It has a worldwide distribution and has been isolated from Europe, Australia, Japan, Hawaii and North America (Schmidt et al., 1979). *V. alginolyticus* was first reported as an opportunistic pathogen of marine animals, however this species is now known to also cause wound and ear infections in humans. This species has been isolated from wound and ear infections but rarely from blood or eye infections (Nishibuchi, 2006). Infections are generally mild and self limiting, lasting only one to two days. Necrotizing fasciitis can also occur but is generally seen in individuals with underlying condition such as cirrhosis. Isolation of *V. alginolyticus* from cases of diarrhea or gastroenteritis is rare and exposure to seawater is considered the primary risk factor for infection by this species. It is still not clear whether all or only some strains of *V. alginolyticus* are capable of causing infections in humans.

1.9.2. Current Assessment of *Vibrio alginolyticus* Infections in the World and in the United States

Between 1997 and 2006, surveillance data from the U.S. indicates that 35% of all non-foodborne vibrio infection was attributable to *V. alginolyticus*. Infections were more likely to affect younger individuals on the Pacific coast where swimming and surfing were frequent exposures (Dechet et al., 2008). Though this species is found to be the predominant *Vibrio* species in many environmental surveys in various parts of the world (Nishibuchi, 2006), the majority of infections are mild and self limiting. Thus, unlike *V. 
cholerae, V. vulnificus and V. parahaemolyticus, this species does not illicit fear and panic among the general public.

1.9.3. *Vibrio alginolyticus* Infections in Hawaii

*V. alginolyticus* infections have been reported throughout the world including Australia, Europe, Mexico, Hawaii and all three coasts of the U.S. when seawater is relatively warm (Blake et al 1980). The majority of infections in Hawaii are due to surfing wounds and coral cuts through which the bacterium gains entry to the body. The actually number of cases may not known because many people do not seek medical treatment due to the self limiting nature of the disease.

1.9.4. Transmission, Pathogenesis, Environmental Factors and Virulence Markers

Skin and ear infections account for most human infections of *V. alginolyticus*. Wound infections are more common among surfers and divers who introduce these bacteria into cuts acquired from coral abrasions. Symptoms of infection include, pain, redness and swelling in the area of the wound. Infections are generally mild and self limiting, however deep-seated or necrotizing soft tissue infections have been reported (Gomez et al., 2003). The route of these infections is direct contact with contaminated seawater or ingestion of raw seafood, which is the same as that of other *Vibrio* infections (Li et al., 2009). Possible virulence factors have been examined in this species such as hemolysins, lipases and collagenases but their role in pathogenesis is still not clear (Nishibuchi, 2006). Furthermore, in wounds, this species is usually found in mixed culture with other microorganisms making it difficult to identify how infection occurs (Schmidt et al., 1979). Generally no treatment or only localized therapy is needed for *V.*
*Alginolyticus* wound infections. This organism is known to be resistant to penicillins and vancomycin, thus ciprofloxacin, tetracycline or chloramphenicol are given to individuals requiring antibiotic treatment (Li et al., 2009). With cases of deep tissue or necrotizing tissue infections surgical debridement may be required.
CHAPTER 2
REVIEW OF LITERATURE: METHODS TO RECOVER HUMAN PATHOGENIC VIBRIO SPECIES FROM RECREATIONAL WATERS AND THE NEED TO DETERMINE THEIR PUBLIC HEALTH SIGNIFICANCE

2.1. CURRENT RECREATIONAL WATER QUALITY STANDARDS ONLY MEASURE RISK FOR SEWAGE BORNE DISEASES

The recreational use of fresh and marine waters for contact water sports (wading, swimming, surfing, canoeing) and non-contact sports (fishing, picnicking) is increasing worldwide. In addition to recreation, bodies of water serve as a source of food and employment. It is believed that currently 50% of the world’s population live in areas within 100 km of the coast and many of these people recreate at the seashore and consume seafood harvested from marine waters (Shuval, 2003). Furthermore, the ease of travel has made access to these areas much easier for larger portions of the population. Increased human dependence on the use of coastal waters coincide with increasing pollution of these water bodies by human pathogenic microorganisms or toxic chemicals and increased health effects to swimmers. In addition to human health effects, pollution results in economic losses when these coastal waters are closed for harvesting of seafood or closed for the many contact recreational uses by tourists and the general public.

2.1.1. Recreational Waters Serve as Vectors for Transmission of Diseases

Bacteria, viruses and protozoan represent three major groups of microorganisms that are pathogenic to man. It is estimated that 120 million cases of gastrointestinal disease are caused by recreational contact of wastewater polluted coastal waters each year (Shuval, 2003). Illnesses are mainly due to swimmer exposure to microbial pathogens which can enter recreational waters through point sources such as sewage outfalls
(Abdelzaher et al, 2010). Table 2.1 is a list of common bacterial, viral and protozoan waterborne pathogens that are found in raw human sewage. The most frequently identified etiologic agents of disease are the Norovirus and Hepatitis A viruses

Table 2.1 Examples of Major Waterborne Pathogens and their Concentrations in Raw Sewage.

<table>
<thead>
<tr>
<th>Pathogen</th>
<th>Disease</th>
<th>Numbers per 100ml</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Bacteria</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Campylobacter</em></td>
<td>Gastroenteritis</td>
<td>$10^4$-$10^5$</td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td>Gastroenteritis</td>
<td>$10^6$-$10^7$</td>
</tr>
<tr>
<td><em>Salmonella</em></td>
<td>Gastroenteritis</td>
<td>0.2-8,000</td>
</tr>
<tr>
<td><em>Shigella</em></td>
<td>Dysentery</td>
<td>0.1-1,000</td>
</tr>
<tr>
<td><strong>Viruses</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hepatitis A</td>
<td>Hepatitis</td>
<td>not enumerated</td>
</tr>
<tr>
<td>Rotaviruses</td>
<td>Diarrhea</td>
<td>400-85,000</td>
</tr>
<tr>
<td>Noroviruses (Norwalk Virus)</td>
<td>Diarrhea</td>
<td>10 infectious particles*</td>
</tr>
<tr>
<td><strong>Parasitic Protozoa</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Cryptosporidium</em></td>
<td>Diarrhea</td>
<td>0.1-39</td>
</tr>
<tr>
<td><em>E. histolytica</em></td>
<td>Amebic dysentery</td>
<td>0.4</td>
</tr>
<tr>
<td><em>G. lamblia</em></td>
<td>Giardiasis</td>
<td>12.5-20,000</td>
</tr>
</tbody>
</table>

* CDC, 2010b.
  (modified from WHO, 2003)

(Debartolomeis & Cabelli, 1991). These viruses require low infectious dose or low numbers of viruses (10-100 viral particles) to cause infection and disease. Other known enteric microorganisms capable of causing gastrointestinal diseases are pathogenic protozoa (*Giardia lamblia*, *Cryptosporidium* spp.) as well as bacterial pathogens (*E. coli* O157:H7, *Shigella* spp., *Salmonella* spp.). The dose required to cause illness is dependent upon several factors, such as, the type and form of microorganisms, and the susceptibility of the host. In this regard, most bacterial pathogens require high infectious doses ($10^6$ to $10^8$ cells). As a result, the risk for transmission of bacterial pathogens by recreational water uses is low. However, a few bacterial pathogens, such as *E. coli* O157:H7, require a low infectious dose (10 to 100 cells) to cause infection and illness
In addition to drinking contaminated water that results in intestinal infection, recreational waters can transmit infections of the upper respiratory tract, ears, eyes, nasal cavity and skin of bathers. These illnesses are often mild and therefore, identification that recreational water exposure was the source of infection has always been difficult to prove.

Besides sewage contamination, human fecal contamination can occur from leaching of septic tanks or from rivers that receive sewage discharges (WHO, 2003). Non-sewage sources of pathogens such as domesticated and wild animal feces and urine are other sources of human pathogens. Humans that enter coastal waters may also be sources of pathogens which may be naturally shed into recreational waters by swimmers which can go on and infect other swimmers. Another problem source for pathogens are those that are naturally found in and capable of multiplying in coastal waters. These pathogens, such as *Vibrio* spp., are difficult to manage because basic knowledge as to expected concentrations in coastal waters, what causes them to multiply and whether they are virulent or non-virulent strains are not known.

**2.1.2. USEPA Recreational Water Quality Standards Only Target Fecal Sources of Pathogens**

Because of the potential for illness from exposure to fecally contaminated recreational waters, a method for the routine analysis of these waters is necessary to assess their level of contamination. Though most gastrointestinal illnesses have been attributed to human enteric viruses methods to detect these viruses are too difficult for routine analysis. USEPA has determined that it is both difficult and expensive to routinely monitor for even the more easily measured pathogenic bacteria. Since
pathogens in recreational waters cannot be monitored, USEPA has adopted a strategy for setting water quality standards based on concentrations of fecal indicator bacteria, or bacteria such as enterorocci and *E. coli*, which are always present in high concentrations in sewage and are easy to measure. (Fujioka, 1988). The current recreational water quality standards established by USEPA were based on the results of epidemiological studies conducted by the agency during the 1970s and were related to numbers of fecal indicator bacteria in water that correlated to acceptable gastroenteritis disease rates in swimmers. For fresh water the acceptable disease rate was established as 8 diseases per 1,000 swimmers, and the standard was set at geometric mean of 126 *E. coli* per 100 ml or 33 enterococci per 100 ml. For marine waters, the acceptable disease rate of 19 out of 1,000 swimmers was established and the standard was set at a geometric mean of 35 enterococci per 100 ml (USEPA, 1986). USEPA guidelines state that a body of water is considered non-compliant if these standards are not met. The agency has forced all states to adopt the current water quality standards, however, some states continue to use previous standards based on concentrations of total coliform or fecal coliform bacteria.

### 2.1.3. Limitations of the Current USEPA Water Quality Standards

USEPA guidelines state that when fecal indicator bacteria exceed the water quality standards these waters are fecally contaminated and may contain dangerous levels of human pathogenic sewage borne microorganisms (WHO, 2003). However, the USEPA guidelines are reliable only if these fecal indicator bacteria meet the following five criteria previously established by scientists: 1. the indicator must be present whenever pathogens are present and should be associated with sewage, 2. the indicator should not be able to multiply in the environment, 3. it should be more resistant to
disinfection than pathogens, 4. there should be a positive correlation between the indicator and health hazards caused by fecal pollution and 5. the assay methods should be easy and rapid. In setting recreational water quality standards, USEPA assumes that the fecal indicator bacteria used meets the five stated criteria. However, currently, even USEPA (Expert Workshop 2007) has agreed that these fecal indicator bacteria do not meet all the criteria and therefore the USEPA recreational water quality standards are not reliable when used to monitor most beaches in the United States. USEPA (Expert Workshop 2007) also acknowledges that water quality standards may not be reliable at beaches in tropical environments where these fecal indicator bacteria have been documented to grow in the soil environment (Fujioka and Byappanahalli, 2003).

Some of the decisions made by USEPA in establishing water quality standards have been criticized. First, the study sites used included in the epidemiological studies conducted by the USEPA to establish water quality standards were all contaminated with sewage discharge and were located in temperate regions of the continental U.S. Second, these water quality standards, based on concentrations of fecal indicator bacteria (enterococci, E. coli), were then applied to all regions (temperate and tropical) of the U.S. Third, although reviews by Prüss (1998) and Wade et al. (2003) have shown significant correlation between the USEPA recommended standard for marine beaches (enterococci) and illness in swimmers when point source pollution occurred, such correlations were not observed when beach waters were contaminated with non-point source pollution (Calderon et al, 1991; Colford et al, 2007; Fleisher et al, 2010). Fourth, USEPA made two unreliable assumptions when fecal indicator bacteria were adopted as indicators of fecal pollution. The first assumption was that the only sources of these bacteria are the
feces of humans and other warm-blooded animals. The second assumption was that these indicator bacteria do not multiply in the environment. Unfortunately, these two assumptions are not applicable to tropical environments such as Hawai’i and Puerto Rico (Rivera et al., 1988; Hazen, 1988) where these fecal indicator bacteria are naturally present and multiply in the environment. Numerous studies (Fujioka et al., 1999; Fujioka & Byappanahalli, 2003; Rivera et al, 1988) have shown that E. coli and enterococci persist in tropical soils and environmental waters. As a result, routine monitoring of waters in tropical areas will lead to consistently elevated levels of these indicator bacteria, which do not represent a health risk because they are associated with environmental (non-point) sources rather than a sewage source. These limitations have led to alternative indicators such as Clostridium perfringens, Male-specific coliphages, and human specific Bacteroides spp. as more reliable indicators of fecal pollution because they are found in high concentrations in human sewage, do not multiply in the environment and are found in low concentrations in recreational waters under ambient conditions (Fujioka & Byappanahalli, 2003; Dick & Field, 2004).

Another limitation of the current USEPA standards is a lack of data to show that elevated concentrations of fecal indicator bacteria in coastal waters can be correlated with the predictable levels of pathogens. This can lead to two erroneous scenarios. The first scenario is, when high concentrations of fecal indicators are found in the absence of pathogens. This is the case in tropical and subtropical areas where non-point sources contribute to high numbers of fecal indicator bacteria. However, non-point sources of pollution present a relatively low-risk source of fecal pollution and therefore may not contain fecal pathogens that may impact human health. Thus, in recreational waters
impacted by non-point source pollution, high fecal bacteria densities may give a false impression that pathogenic microorganisms are present. The second scenario is when pathogens are present in the absence of fecal indicator bacteria. The effectiveness of using coliforms as indicators has come into question because most enteric viruses (Poliovirus, Coxsackie virus, Hepatitis virus, Norwalk virus) are able to survive wastewater chlorination better than most bacteria. Therefore, the absence of these indicator bacteria may not necessarily mean that pathogenic viruses, and more resistant pathogenic bacteria, are absent. Enteric viruses have been recovered from water environments that meet bacterial indicator standards, and there have been virus related outbreaks linked to ingestion of waters that met fecal coliform standards (Craun, 1991). This lack of correlation between fecal indicator bacteria and the presence of pathogenic microorganisms show that recreational water quality standards are unreliable predictors for adverse health effects on bathers (Abdelzaher et al., 2010).

A serious limitation of the current USEPA fecal indicator bacterial standards is that they fail to address pathogens, whose source is not feces. In this regard, swimming related illnesses still occur in the absence of fecal contamination in waters that meet USEPA recreational water quality standards. *Staphylococcus aureus* is pathogenic bacteria whose normal habitat is the skin of humans and this bacteria is shed into waters by bathers. Elmir et al (2007) reported correlations between concentrations of *S. aureus* in coastal waters and infections (skin, eye, ear) among swimmers. The problem of pathogenic *Vibrio* species in coastal waters is more difficult to assess because *Vibrio* sp. are marine bacteria and therefore their normal habitat are coastal waters. In summary,
currently USEPA has not developed or addressed water quality standards for pathogens such as *S. aureus* or pathogenic *Vibrio* spp.

2.2. RATIONALE FOR NEED TO MEASURE QUALITY OF RECREATIONAL WATERS SO RISK FOR TRANSMISSION OF *VIBRIO* SPP. INFECTIONS CAN BE DETERMINED

2.2.1. Evidence that Human Uses of Recreational Waters Can Result in Transmission of Vibrio Infections

Halophilic marine *Vibrio* bacteria are found in coastal waters, estuaries, and marine animals throughout the world, and are one of the most common bacteria found in marine waters (Howard and Bennett, 1993). Four *Vibrio* spp. (*V. vulnificus*, *V. parahaemolyticus*, *V. alginolyticus*, *V. cholerae*,) are pathogenic and can infect humans by ingestion or by causing wound infections following exposure to recreational waters. Of these four pathogenic *Vibrio* spp., *V. cholerae* has a long history of causing many epidemics and pandemics, resulting in many human infections and deaths. However, *V. cholerae* is a disease of poor sanitation and therefore, cholera as a disease is no longer a disease of concern in the U.S. Moreover, the primary mode of transmission of *V. cholerae* is via contaminated drinking water. Although *V. cholerae* has been recovered in coastal water environments in the U.S., there is no evidence for transmission of *V. cholera* via exposure to recreational use of coastal waters in the U.S. In summary, although *V. cholera* is a disease with high severity, in the U.S, transmission of this disease is considered unlikely via exposure to recreational uses of coastal waters.

In contrast, data from CDC show that substantial evidence for transmission of *V. vulnificus*, *V. parahaemolyticus*, and *V. alginolyticus* via ingestion or contact with recreational uses of coastal waters in the U.S. Wound infections and diseases caused by
these three *Vibrio* spp. are considered serious because of the potential for blood infection and related complications. During recreational and occupational exposures to marine waters and seafood products, the unbroken skin of humans generally resist vibrio infection. However, almost all persons who develop vibrio wound infections do so following infection at a skin site which has been previously broken (a wound) by some activity which results in abraiding or penetrating the skin (Oliver, 2005). These infections can result in mild disease symptoms like those caused by *V. alginolyticus*, or they can lead to severe life threatening disease like those caused by *V. vulnificus*. It should be noted that in most wound infections by *V. vulnificus* which lead to death, the patients were determined to be unusually susceptible because their immune systems were compromised by other health problems (Bonner et al, 1983; Oliver, 2005).

In summary, vibrio wound infections have been reported from many countries (North America, South America, Europe, Asia, Austria). However, vibrio wound infections have been reported most frequently in Florida than anywhere else in the world (Howard and Bennett, 1993; Hlady and Klontz, 1996). A surveillance conducted by the CDC from 2005-2006 for waterborne disease outbreaks associated with recreational water exposure reported that the most commonly reported species of vibrios were *V. vulnificus*, *V. alginolyticus* and *V. parahaemolyticus* (MMWR, 2003). These results provide clear evidence that the risk of infection by these vibrios, associated with recreational uses of coastal waters, should be addressed.

### 2.2.2. USEPA Has Not Established a Program to Assess Risk and to Set Guidelines for Presence of *Vibrio* spp. in Coastal Waters.

Although there is enough definitive data to show that coastal waters serve as sources and vectors for the transmission of diseases caused by pathogenic *Vibrio* spp.,
USEPA has not developed a program to assess risk for monitoring coastal waters for *Vibrio* spp. As a result there are no U.S. regulations requiring state, county and private agencies to monitor coastal waters for pathogenic *Vibrio* spp. It is well known that without legislative mandates such as regulations, federal, state and county agencies do not have responsibilities to assess risk for transmission of pathogenic *Vibrio* spp. via recreational uses of water. Under these conditions, funding for programs to initiate monitoring coastal waters for pathogenic *Vibrio* spp. cannot be justified.

### 2.3. ASSESSMENT OF CULTURE BASED METHODS TO RECOVER AND ENUMERATE PATHOGENIC *VIBRIO* SPP. FROM RECREATIONAL WATERS

#### 2.3.1. Use of Growth Media to Culture *Vibrio* spp.

As stated previously, USEPA has not approved a culture based method to recover and enumerate vibrio bacteria from environmental water samples. In this regard, most clinical laboratories do not routinely use culture medium with elevated concentrations of NaCl, which is required to recover most *Vibrio* spp. As a result, most clinical laboratories do not have sufficient data to assess for the presence of *Vibrio* spp. in human clinical samples. To selectively culture *Vibrio* spp. growth media are made selective by addition of selective agents such as NaCl, bile salts, teepol, tellurite and colistin. In addition, pH of the growth medium can be made alkaline (above pH 8) as another selective agent to detect the growth of *Vibrio* spp. (Gomez-Gil and Roque, 2006). If *Vibrio* species are at low levels, an enrichment step may be required prior to plating on selective agar. For the enrichment of *V. cholerae, V. vulnificus* and *V. parahaemolyticus*, alkaline pepetone water (APW) has been widely used and is recommended for use by the USFDA Bacteriological Analytical Manual (BAM) (DePaola and Kaysner, 2004). APW
has a high pH (8.5) and contains NaCl (1%) which inhibits many other bacteria in favor of *Vibrio* species. Water and seafood samples are generally enriched for 3 to 5 hours (or overnight) at 35°C prior to plating on selective agar to recover *Vibrio* spp. Several agar media are available for the isolation of *Vibrio* species, however, thiosulfate citrate bile salt sucrose (TCBS), modified cellobiose polymyxin B colistin (mCPC) and cellobiose-colistin (CC) agars are selective agars recommended by the USFDA for use in the isolation of these species (DePaola and Kaysner, 2004).

### 2.3.2. TCBS Agar as a Culture Medium.

This agar is the most widely used medium to isolate *Vibrio* spp. from clinical, environmental and food samples and is commercially available. Though it was originally designed for the isolation of *V. cholerae* and *V. parahaemolyticus*, most vibrios are capable of producing large, healthy colonies (Gomez-Gil and Roque, 2006). The main inhibitory agent contained in this media is bile salts which inhibit Gram positive bacteria. *Vibrio* spp. are differentiated on this medium based on sucrose fermentation. Sucrose positive species (*V. cholerae*, *V. alginolyticus*) form yellow colonies while sucrose negative species (*V. vulnificus*, *V. parahaemolyticus*) form green colonies on this media. Though TCBS is widely used there are two major drawbacks with this agar. First, acids produced by sucrose-fermenting bacteria diffuse into and spread though the agar with time, changing the color of the agar from green to yellow in the area surrounding the colonies of sucrose-fermenting bacteria or even across the entire area of the agar plate. When sucrose non-fermenting colonies, like *V. parahaemolyticus*, appear near sucrose-fermenting bacterial colonies they are covered by the yellow color leading to difficulties in the differentiation of sucrose negative colonies from sucrose positive ones (Hara-Kudo
et al., 2001). Second, TCBS has been shown to be inhibitory to *V. vulnificus* growth (West et al., 1982) and other marine *Vibrio* species (Harris et al., 1996). Furthermore, a study conducted by Hoi et al. 1998 showed that this agar gave a very low plating efficiency of 1% for both clinical and environmental strains of *V. vulnificus*. As a result, less inhibitory agars such as mCPC and CC were developed.

2.3.3. mCPC Agar as a Culture Medium.

The use of cellobiose-polymyxin B-colistin (CPC) agar has been proven to be successful in the isolation and differentiation of *V. vulnificus* (Massad and Oliver, 1987). This medium takes advantage of the resistance of *V. vulnificus* to colistin and polymyxin B. In addition, high-temperature incubation, at 40°C, inhibits the growth of many marine bacteria, and the fermentation of cellobiose acts as a further differential criterion (Hoi et al., 1998). Colistin and polymyxin B are bacteriocidal agents that interfere with the structure and function of the outer and cytoplasmic membranes (Gomez-Gil and Roque, 2006). A modified version of this agar (mCPC) with a reduced concentration of colistin was reported to be effective for the isolation of *V. vulnificus* from environmental sources (Tamplin et al., 1991). This medium is not sold commercially and has to be made from scratch which may lead to variability within batches of prepared media and among laboratories.

2.3.4. CC Agar as a Culture Medium

*V. vulnificus* strains were found to have a high degree of variation in their sensitivity to colistin. The formulation of cellobiose-colistin agar is similar to mCPC agar with the omission of polymyxin B, making it less selective. Studies have shown that
this medium is superior to selective agar media previously used (CPC, mCPC) for the isolation of *V. vulnificus* (Hoi et al., 1998) and other *Vibrio* species (Macian et al., 2000). Recovery rates of *V. vulnificus* on CC agar were 100%, and 76% and 93% for CPC and mCPC agars, respectively. Hoi et al also showed that *V. vulnificus* was inhibited with increasing concentrations of colistin and polymyxin B. One drawback of this media however is that the reduced concentrations of antibiotics does not inhibit completely the competing and interfering *Vibrio* species (Macian et al., 2000).

### 2.3.5. CHROMagar Vibrio (CV) as a Culture Medium

Several other media have been developed for the isolation of *Vibrio* species. These include taurocholate tellurite gelatin agar for the isolation of *V. cholerae* and bromothymol blue teepol agar for the isolation of *V. parahaemolyticus*. These agars contain the inhibitory agents tellurite and teepol, respectively, which inhibit the growth of background organisms. A relatively new media called CHROMagar Vibrio (CV) is a differential media that is able to distinguish colonies of *V. cholerae*, *V. vulnificus*, *V. parahaemolyticus* and *V. alginolyticus* based on color. It is composed of peptone, yeast extracts, salts, agar and a proprietary chromogenic mix. On this medium, *V. cholerae* and *V. vulnificus* appear blue/turquoise, *V. parahaemolyticus* appears mauve, and *V. alginolyticus* appear as colorless colonies. CV can differentiate between *V. vulnificus* and *V. parahaemolyticus* unlike on TCBS where they both do not ferment sucrose and appear green. Also, unlike with TCBS, where sucrose positives can cover sucrose negative colonies, colors on CV are not affected by the presence of colonies of other bacteria on the same plate, because generation of the color depends on the reaction of the bacterial beta-galactosidase with the substrate contained in the media. It has also been shown that
CV, with respect to *V. parahaemolyticus*, had a higher rate of detection than when TCBS was used (Hara-Kudo et al., 2001). The advantage of using CV medium is that it is commercially available and has been used extensively to recover *Vibrio* spp. from human clinical samples. However, more studies are needed to determine the reliability and feasibility of using this medium to recover human pathogenic *Vibrio* spp. from coastal water samples.

2.4. ENVIRONMENTAL FACTORS THAT CONTROL THE VIABLE CONCENTRATIONS OF *VIBRIO* SPP. IN ENVIRONMENTAL WATERS

Numerous studies have been conducted to determine the relationship between *Vibrio* spp. abundance and environmental factors such as temperature, salinity, nutrients, and dissolved oxygen. As a result, these water quality characteristics can be used in a predictive manner to determine when these pathogens may be present. From these studies, the two most significant factors that affect the ecology of *Vibrio* spp. are water temperature and salinity. Understanding the relationship between concentrations and prevalence of *Vibrio* spp. in coastal waters and the role of temperature and salinity are the most likely factors to predict their concentrations in shellfish and recreational waters, and thus provide a way to make public health management decisions.

2.4.1. Water Temperature

Water temperature is a key factor in the ability to isolate pathogenic *Vibrio* spp. All human pathogenic *Vibrio* species are mesophilic (20-40°C) though there are cold adapted *Vibrio* spp. that can grow below 4°C (Urakawa and Rivera, 2006). Early studies of *V. cholerae* in the Chesapeake Bay showed that this microorganism could not be isolated from the Bay when water temperatures dropped in the winter below 10°C.
Additional studies in temperate regions of the world have shown that *Vibrio* spp. are frequently detected by culture during the summer months when water temperatures exceed 15°C, but are less common during the winter months when temperatures drop below 15°C. For example, studies with *V. vulnificus* have shown that this species is generally recovered when water temperature range between 15-32°C (Veenstra et al., 1994; Tamplin et al., 1982) and cannot be isolated when water temperatures drop below 15°C (Pfeffer et al., 2003). These studies and others (Randa et al., 2004) have concluded that water temperature can account for almost 50% of the variability in *V. vulnificus* abundance, making temperature a major factor in determining the seasonal dynamics of this species. *Vibrio* spp. are also recovered more readily from shellfish during the warm water months and are correlated with incidences of human infection. Of the *V. vulnificus* infections reported in the U.S. from 2000-2003, 85% of cases occurred from May to October when water temperatures were warmer (Oliver, 2005). Similar temperature-mediated dynamics have been shown in several other species of *Vibrio*. For example, the abundances of *V. cholerae* and *V. parahaemolyticus* have also shown seasonal fluctuations in estuaries and coastal waters (Louis et al., 2003; DePaola et al., 2003, Deter et al., 2010). In tropical and subtropical areas, such as Hawai‘i, such seasonal variation is low. Because Hawaii’s water temperatures are generally between 24-27°C year round, *Vibrio* spp. have been shown to be present throughout the year.

One hypothesis why *Vibrio* spp. are not recovered by culture when water temperature falls below 15°C is because these bacteria may go into a viable but non-culturable (VBNC) state. The VBNC state has been described for over 100 species of bacteria in over 30 genera (Oliver, 2005). In this state, cells lose the ability to be cultured
on routine media, but fluorescent assays conducted directly on water samples show that these vibrios are present in a dormant state in waters throughout the year (Roszak and Colwell, 1987). Furthermore, the cells can retain viability, and in many cases have been shown to be capable of resuscitation to an actively, metabolic state. This was demonstrated in situ in an estuarine environment through the use of membrane diffusion chamber (Oliver at al., 1995). In this study, when water temperatures fell below 15°C \( V.\) \textit{vulnificus} cells stopped growing on routine media but were still alive as indicated by direct viable counts. This study also demonstrated that these cells became resuscitated during warm weather months when water temperatures were between 22 and 24°C. Resuscitation of \textit{Vibrio} species is controlled by the temperature sensitive activity of catalase, which is required by cells to detoxify hydrogen peroxide naturally present in routine laboratory media, thus, at higher temperatures there is higher cellular catalase activity (Oliver, 2005).

The second hypothesis states that the population of \textit{Vibrios} overwinters in the flocculation zone at the sediment-water interface, and in early spring, the cells become resuspended into the water column by wave action. A study (Randa et al., 2004) of \( V.\) \textit{vulnificus} in Barnegat Bay showed that cells remained elevated in sediment during the winter and mixing events during the spring resuspended these cells from the sediment, resulting in elevated numbers of viable cells in the water column. Thus, this process may be a mechanism by which the water column is seeded with \( V.\) \textit{vulnificus} during the spring, leading to high counts of this bacterium in the summer when temperatures are favorable for their growth. In this regard, studies have shown that \textit{Vibrio} spp. overwinter in temperate waters by growing as biofilm in copepods during winter months when the
temperature of water is unsuitable for growth. These results show that Vibrio spp. grow as biofilm on many surfaces in the marine environment. The significance of vibrio growth as biofilm on sediments, on marine organisms and on marine plants remains to be determined.

2.4.2. Water Salinity

Salinity is the second important environmental factor that has been shown to influence the abundance of different Vibrio spp. As a result, the identification of Vibrio species is accomplished by using a test to determine the growth range in different concentrations of Na\(^+\). Vibrio spp., like most marine bacteria, require sodium ions for growth. Sodium ion is required by sodium proton antiporters in the energy transducing cytoplasmic membrane. Na\(^+\)/H\(^+\) antiporters are membrane proteins that play a major role in pH and Na\(^+\) homeostasis of cells. Moreover, other inorganic ions (calcium, magnesium) are required to maintain the integrity of the cell wall and membrane of marine bacteria.

Freshwater discharges from surface streams, storm drains and from groundwater flow can affect coastal water salinity and can influence which species of Vibrio are found. For example, V. cholerae has low salinity requirements and is able to survive for long periods of time in fresh and brackish waters and is rarely recovered from open ocean water, which are characterized by high salinity. The optimal salinity range for V. cholerae was shown to be between 2 and 14 parts per thousand (ppt) in one study (Louis et al., 2003), which is in agreement with previous studies done in Chesapeake Bay where V. cholerae was isolated at salinities ranging from 2 to 14 ppt (Louis et al., 2003). Furthermore, studies in Southern California found that high concentrations of V. cholerae
were more frequently detected at salinities below 10 ppt but above 0 ppt (Jiang, 2001). In fresh waters or at high salinities the growth of *Vibrio* spp. as biofilm on zooplankton may explain the occurrence of *V. cholerae* at these sites where conditions are unfavorable for their growth (Louis et al., 2003).

Unlike *V. cholerae*, *V. vulnificus*, *V. parahaemolyticus* and *V. alginolyticus* have higher salinity requirements and are generally not found in freshwater and low salinity water bodies. These *Vibrio* spp. are found in marine waters ranging from estuaries, to coastal and open ocean waters. In this regard, *V. vulnificus* does not appear to survive well at elevated salinities and has not been isolated from open ocean waters, but is mostly isolated from water with salinities between 8 and 23 ppt (Oliver, 2005). Furthermore, studies have shown that salinity was the controlling factor of *V. vulnificus* concentrations in oysters from the Gulf and Atlantic Coast where levels of this species were highest (10^3/g) in oysters harvested at salinities between 5 and 25 ppt (Motes et al., 1998). Studies on *V. parahaemolyticus* show that this species can be recovered in lower salinity waters when salinities are greater than 15ppt (Kelly and Stroh, 1988). However, other studies (DePaola et al., 1990; Kaneko and Colwell, 1975a) suggest that the *V. parahaemolyticus* is less salinity dependent than *V. cholerae* and *V. vulnificus*. Nonetheless, it has been suggested that once permissive water temperatures are reached, salinity becomes the controlling factor in the occurrence of this species in the environment (Kelly and Stroh, 1988). Of the four *Vibrio* spp., *V. alginolyticus* has the highest salinity tolerance. This species is generally found in high salinity coastal waters and is able to tolerate 10% (wt/v) sodium chloride, which is a feature that can be used to
distinguish this species from *V. cholerae*, *V. vulnificus* and *V. parahaemolyticus* (DePaola and Kaysner, 2004).

### 2.4.3. Other Environmental Factors (Water Nutrients, Sunlight)

Other environmental factors that have been studied with regard to *Vibrio* spp. prevalence are turbidity, pH, nutrients and sunlight. Some studies have shown a positive correlation between *V. vulnificus* and turbidity and pH of the water (Oliver et al., 1982; Oliver et al., 1983) while others have not found an association (Pfeffer et al., 2003). Waste contamination and land runoff may contribute directly as sources of *Vibrio* spp. or indirectly by contributing nutrients needed by vibrios or providing nutrients to other marine organisms, which are then used by *Vibrio* spp. (Tamplin et al., 1982). Fluxes of land based runoff as freshwater are known to provide nutrients, dissolved organic and inorganic matter as well as to increase turbidity and decrease salinity. Therefore, salinity may be a proxy for other factors such as turbidity and nutrient load (Louis et al., 2003). Negative correlations have also been seen with dissolved oxygen levels and *V. vulnificus* species abundance (Pfeffer et al., 2003) which is reasonable given that water temperature and dissolved oxygen levels are negatively correlated and that water temperature plays a significant role in the distribution of this organism (Oliver, 2006). The presence of host organisms (copepods, crabs) may select for certain *Vibrio* spp. which are capable of deriving nutrients from the chitinous exoskeletons of these animals (Kaneko and Colwell, 1975b; Sochard et al., 1979; Davis and Sizemore, 1982). In some studies (Abboudi et al., 2008) exposure of vibrio bacterial cells to natural sunlight have been reported to cause damage to nucleic acids and proteins via the production of reactive oxygen species. Moreover, careful experiments have shown that sunlight exposure of *V. cholerae* can
activate lysogenic phage (CTXΦ) to induce the propagation of toxin producing cholera strains (Faruque et al., 2000). Thus, it is possible that sunlight may be a natural factor that may play a role in the initiation of cholera epidemics.

2.5. MOLECULAR BASED METHODS TO SPECIATE AND TO DETERMINE VIRULENCE MARKERS IN VIBRIO SPP.

2.5.1. Advantages of Molecular Methods to Detect Pathogenic Vibrio spp.

Outbreaks of illness related to exposure to marine waters and marine foods have heightened the need to develop rapid and reliable methods to detect pathogenic Vibrio species in shellfish and swimming waters. Detection of Vibrio spp. by culture methods is the only acceptable way to monitor waters for concentrations of Vibrio spp. However, culture methods are labor intensive, time consuming and there are limitations in detecting all relevant species of Vibrio. To address the limitations of culture methods, some commonly used culture independent techniques such as, fluorescent in situ hybridization (FISH) assays, colony hybridization and quantitative polymerase chain reaction (QPCR) have been developed. The use of these methods is especially promising because they are rapid and can be made very specific to the species of Vibrio. Targets for these methods often assay for specific genes of microorganisms such 16s rRNA or specific virulence genes for each species of Vibrio. In addition, these methods may be important for determining Vibrio abundance when they are in a dormant state (VBNC) when culture methods are ineffective.
2.5.2. FISH Assay Method

Fluorescent in situ hybridization (FISH) with rRNA targeted probes is a staining technique that allows phylogenetic identification of bacteria in mixed assemblages, without prior cultivation, by means of epifluorescence or flow cytometry (Pernthaler et al., 2001). Nucleic acids from water, tissue and sediment samples are extracted and hybridized with oligonucleotide probes targeting 16s rRNA. FISH assay have been used to shed light on the distribution and ecology of vibrios in the marine environment and have overcome the problem of the great plate anomaly, i.e. the difference of $10^2$-$10^3$ between direct cell counts and CFU counts. Drawbacks of this method include low fluorescence intensity of marine bacteria and the fact the several Vibrio species have similar 16s rRNA sequences, making it difficult to perform reliable species identification (Thompson et al., 2004).

2.5.3. Colony Hybridization Method

Colony hybridization is not exclusively a molecular method, but is a combination of plate count and confirmation of the colony through DNA hybridization (Gomez-Gil and Roque, 2006). Most hybridization uses species-specific probes based on variable regions of the 16s rRNA. This method has been commonly used for the detection of V. vulnificus in environmental samples. Wright et al (1993) evaluated this method for the detection of V. vulnificus using an alkaline phosphatase-labeled oligonucleotide probe derived from a DNA sequence with its hemolysin gene. In this study, results obtained from colony hybridization positively correlated with other detection methods used. This method has also been used to enumerate and confirm the presence of pathogenic strains of V. parahaemolyticus using probes that target genes $tl$ (total V. parahaemolyticus) and
tdh and trh (pathogenic V. parahaemolyticus) (DePaola et al., 2000 and 2003). The main advantage of colony hybridization is that it allows the rapid isolation and enumeration of Vibrio spp. without enrichment or the use of selective media (Wright et al., 1993), however probes for some species can cross react with other species leading to erroneous results (Thompson et al., 2004).

2.5.4. Quantitative Polymerase Chain Reaction (QPCR) Method

QPCR can be used to enumerate Vibrio species in environmental samples. This method is based on the detection and quantification of a target sequence/gene using a fluorescent reporter whose signal increases in direct proportion to the amount of target that is present in the sample. As a result, the higher the starting copy number of the nucleic acid target in the sample, the sooner the fluorescence will be measured (Gomez-Gil and Rooque, 2006). TaqMan probes are most commonly used when detecting Vibrio species such as V. cholerae, V. vulnificus and V. parahaemolyticus. Assays using these probes are available for the quantification of V. vulnificus in seawater and oyster meat (Takahashi et al., 2005). The ToxR gene (toxR) has been commonly used as a target to detect different Vibrio species using QPCR. It is a housekeeping gene present in Vibrio spp., though partial sequences are different among species (Takahashi et al., 2005). One advantage of using QPCR technology over culture is that these methods are much faster, thus results regarding concentrations and the presence of virulence genes can be obtained more rapidly. However, because both dead and live cells are measured no conclusions about infectivity can be made. As a result, results from molecular methods cannot be used to make reliable risk estimates for human infection. In summary, from a public health standpoint, molecular methods are not acceptable methods. Furthermore, environmental
samples provide other limitations. First, there are interfering substances in environmental samples that can lead to QPCR inhibition. Second, some environmental microorganisms, which differ from *Vibrio* spp., may have the same gene sequence as the targeted *Vibrio* spp.

2.6. METHODS USED TO ASSESS RISK BASED ON DETECTION AND ENUMERATION OF PATHOGENS AND PATHOGEN INDICATORS IN RECREATIONAL WATERS

2.6.1. Expectations and Goals of Government Agencies to Ensure that Water is Safe for Public Use

The basic role of public health is the prevention of disease and the promotion of health within a community. This is accomplished by identifying health risks in a community, detecting and preventing the spread of disease, promoting healthy lifestyles, and informing the public of health issues. Once risks to health are identified, government can develop policies and regulations that will safeguard the public. Public concern over environmentally related health effects has shaped the evolution of public policy in the U.S. Unfortunately, the ability of health professionals to adequately respond to this concern has often been limited by funding and by limited technology (Aldrich et al., 1993).

With regard to water quality, U.S. federal and state agencies have adopted regulations to ensure that the population is protected from ingesting waterborne pathogens either through drinking or recreational waters. The intended use of the water is an important factor to consider when regulations are put into place. For example, drinking water regulations are much more stringent than those for recreational waters. This is because drinking water is used by all people within a community while only a
portion of a community will come into contact with recreational waters. Thus, contaminated drinking water has the potential to adversely affect a larger segment of a population as compared with recreational waters. When contamination events occur governmental agencies must step in to safeguard the public. For example, if elevated levels of indicator bacteria are present in a swimming site, the local government will have to close the site to bathers until indicator levels are acceptable. If local agencies fail to protect the public they may face heavy fines imposed by the federal government and lose the trust of the public they are trying to protect.

While water quality guidelines have been established for fecal pollution no such guidelines exist to protect the public from pathogens that are naturally present in recreational waters. Currently there are no standards available for Vibrio spp. either for recreational waters or for shellfish harvesting waters. Shellfish such as oysters are known to be a significant source of Vibrio infections, however a fecal coliform standard of 14 MPN per 100 ml of shellfish raising water is currently used to assess the quality of harvesting beds. While this standard may indicate when pathogens related to human fecal inputs are present, they do not indicate when naturally occurring pathogens are present. Thus, for some pathogens (such as vibrios) the primary option available to public health managers may be to develop educational material and communicate risk in areas where these pathogens are known to reside (Pond, 2005).

2.6.2. Definition of Risk

According to the USEPA, “risk” is defined as the chance of harmful effects to human health or to ecological systems resulting from exposure to an environmental stressor. A stressor can be a physical, chemical, or a biological entity that can induce an
adverse response. Risks can range from adverse events that occur at low frequency to more regular events that can occur more frequently. There are several classes of hazards that can impact individuals who recreate in fresh and coastal waters. The first class are physical hazards at the beach site such as water depth, water current, rocks or buoys

Figure 2.1. Schematic approach to comparing health hazards encountered during recreational water use (from WHO, 2003).

which can lead to accidental drowning. The second class are physical and chemical conditions of the water such as water temperature, pH, salinity, sunlight, dissolved oxygen, turbidity and nutrient load that control survival and growth of pathogenic microorganisms such as *Vibrio* spp. The third class are biological factors, especially
pathogenic microorganisms (e.g. *Vibrio* spp.) which can infect humans and cause diseases. The fourth class are chemical factors such as chemical toxins, heavy metals, nutrients, and antibiotics, which can affect humans directly or can make pathogens more virulent (WHO, 2003). Figure 2.1 is a comparison of health hazards encountered during recreational water use.

A severe health outcome (drowning, spinal injury) that impacts only a small number of individuals may require high management priorities while minor health problems (skin irritations) that affect a larger number of individuals, but do not result in incapacitation, may require lower management priorities (WHO, 2003). Of these hazards, a lot of attention has been focused on microbial hazards in recent years particularly health risks associated with contact with waters contaminated by sewage. This is because the potential for human disease associated with exposure to waterborne pathogenic microorganisms has become a growing public health concern and is related to human pollution events that can be controlled (ILSI, 2000). Since pollution of coastal waters by pathogens result in placing large numbers of individuals at risk several methods can be used to determine risk to swimmers.

### 2.6.3. Methods to Assess Risk

#### 2.6.3.1. Epidemiological Method: Considered the Best Method to Determine Risk Because it Measures Disease Rate in a Human Population

Epidemiology is defined as the study of the occurrence and distribution of disease and injury specified by person, place, and time (Griffith and Aldrich, 1993). Epidemiological studies are central to the assessment of risk by providing estimates of risk and data for risk assessment models (Pond, 2005). They can yield more defensible
estimates of likely human health risks than those from biological models based on animals studies (Nurminen, 1999). Epidemiologists evaluate disease patterns in communities to identify pathogens with high, medium or low risk and their mode of transmission. Once these characteristics are determined, epidemiologists recommend the most effective mitigation means to prevent further disease occurrence (Griffith and Aldrich, 1993). Observational epidemiological studies are the most common type of study used and include cohort, case control and cross sectional studies. Cohort studies are the most commonly used epidemiological studies to assess health risks associated with human contact and exposure of recreational waters. In cohort studies, the study population is initially free of illness and is divided into subpopulations (i.e. those who come into contact with water vs. those who don’t). Individuals are then followed up, at a later date, and questioned regarding disease symptoms (i.e. diarrhea, skin rash). With case control studies the selected study population is known to have the illness of interest, and epidemiologists look back in time in order to associate the illness with exposure to a particular factor. Finally, cross sectional studies reflect a “look and see attitude” (Griffith et al, 1993) where both the exposure and disease are determined simultaneously. Because of this, it is not possible to determine if exposure precedes or follows the onset of disease, and thus do not show a strong cause-effect relationship.

In transmission of water borne diseases epidemiological studies are considered the gold standard for providing reliable risk estimates based on exposure. Epidemiological studies conducted by the USEPA have been used to establish the recreational water quality standards that are in place today. In the studies, gastrointestinal disease rates were measured in exposed populations and compared with unexposed
populations. The results showed a definite correlation between exposure to recreational uses of water and degree to which these waters were contaminated with sewage effluent. However, in these studies, the etiological agent that caused disease among the swimmers was not determined. Based on the incubation period and the disease symptoms, USEPA concluded that human enteric viruses, such as norovirus, were the most likely etiological agent. Epidemiological studies are expensive and a demanding study design. Results are valid only when large numbers of subjects are used in order to attempt to negate biases and control for confounding factors that are present. Biases such as misclassification of data or interviewer bias can skew results in a particular direction. Confounding factors are variables that may cause the same effect to the study population as that of the risk factor being investigated, thus showing associations between the risk factor and the exposed population that are not really present. Therefore, epidemiological studies have to be constructed carefully in order to obtain sound and useful data.

2.6.3.2. The Risk Analysis and Assessment Method: A Comprehensive Approach to Predict and Communicate Risk

The epidemiological method cannot be applied to all identified risks, including risk for infection by pathogenic Vibrio spp. The increase in public health concern over human disease associated with waterborne microbial pathogens has heightened the awareness and need for the development of methods to assess the risk of human disease from waterborne pathogens (ILSI, 2000). Risk analysis brings logic, reason, and scientific deliberation to bear on hazard management (Slovic et al., 2004). It includes three elements: risk assessment, risk management and risk communication. Risk assessment is a process that evaluates the likelihood that adverse ecological or human
health effects will occur as a result of exposure to stressors. It involves hazard identification, analysis of the linkage between exposure to hazards and effects on receptors (i.e. swimmers), and risk characterization (USEPA, 1998). Risk assessments are used to help risk managers determine priorities for actions that are designed to manage or reduce risk. Assessments of health impacts of recreational water quality are
increasingly being used as a scientific rationale for developing appropriate policies for risk management (Pond, 2005).

Risk assessments generally involve three major parts: (1) problem formulation, (2) analysis (characterization of exposure and human health effects), and (3) risk characterization. The USEPA ecological risk assessment outline is shown in Figure 2.2. Prior to the problem formulation phase, risk assessors and risk managers must discuss several points. The first is to set management goals which are statements about what the (ecological) need is (i.e. prevent fecal contamination of recreational waters). These goals drive the risk assessment and are often based on laws that are already in place (i.e., the Clean Water Act). Once these goals are set, managers must discuss what options are available to achieve these goals (i.e. treatment of sewage influent before discharge). Finally, managers must determine how much resources (time, finances) are available to conduct the risk assessment. The four major parts of risk assessment are summarized below.

**Problem Formational Phase.** This phase is the foundation of a risk assessment. During this phase risk hypotheses or assumptions are generated about why ecological effects have occurred. The stressor and the specific entity to be protected are identified and a conceptual model is prepared showing the predicted relationship between the stressor, exposure and the impact on the ecological receptor (i.e. humans). A plan is then developed to analyze data and characterize risk.

**Risk Analysis Phase:** This phase consists of the technical evaluation of the data concerning the potential exposure and the associated health effects, and is based on the conceptual model developed during the problem formulation phase (ILSI, 2000). This
phase involves assessing exposure (exposure characterization) and human health effects. Exposure characterization involves assessing the interaction between the pathogen, environment and humans, and three elements are involved: pathogen characterization, pathogen occurrence and exposure analysis. Pathogen characterization involves determining the qualities of the pathogen that permit it to be transmitted to and cause disease in a host. These include virulence factors, resistance to treatment processes, survival and multiplication in the environment and ecology. The specific characteristics that are evaluated are dependent on the scenario outlined during the problem formulation phase and the biology of the organism (ILSI, 2000). Pathogen occurrence involves determining such pathogen characteristics as, their sources, distribution and concentration in the environment, seasonality and spatial distribution (clumping, aggregation) in the environment. The physical state of the pathogen can affect its state and occurrence in the environment. Pathogens that bind to aggregates, for example, may be protected from environmental control measures and lead to higher exposure in humans. The outcome of pathogen occurrence is an evaluation of all relevant factors pertaining to the occurrence and distribution of the pathogen. Exposure analysis involves identifying the source and temporal nature of human exposure to pathogenic microorganism. Information that may be included in exposure analysis includes the vehicle of pathogen transmission (i.e. drinking water) units and routes of exposure and the size and demographics of the exposed population. Characterization of exposure culminates in the development of an exposure profile that quantitatively or qualitatively evaluates the magnitude, frequency and pattern of human exposure for the scenario developed during problem formulation and serves as an input for risk characterization (ILSI, 2000). The exposure profile takes
into account data from pathogen characterization, pathogen occurrence and exposure
analysis as well as uncertainties and limitations encountered during this phase of risk
analysis. These limitations are then considered during the risk characterization phase.

The human health characterization phase of risk analysis involves characterizing
the host, evaluating the human health effects and determining the dose-response
relationship between the pathogen and the host. The overall objective of host
characterization is to identify susceptible subpopulations and the factors that influence
their susceptibility to illness by a particular pathogen. Susceptibility is the extent to
which a host is vulnerable to infection by a pathogen, taking into account a hosts’
intrinsic and/or acquired traits that modify the risk of infection or illness (ILSI, 2000).
Such factors as age, immune status and genetic background of a population can affect
how susceptible that population is to infection. For example, high risk groups such as the
very young or old, those who are pregnant, or those who are immunocompromised may
develop more severe, symptomatic illness whereas low risk groups may develop mild
illness or be asymptomatic. Evaluating the human health effects of a pathogen involves
evaluating the clinical illness associated with the pathogen. This includes looking at the
duration and severity of illness, the infectivity of the pathogen and the extent of
secondary transmission. Typically this kind of information can be derived from
epidemiologic and clinical studies. These types of studies provide the opportunity to
obtain data in a natural setting and have the ability to assess susceptible subpopulations,
seasonality of the pathogen and secondary transmission. The third component of human
health characterization is determining the dose-response relationship between a pathogen
and its host. Dose-response analysis is used to characterize the relationship between
pathogen dose, infectivity and manifestation and magnitude of health effects in an exposed population (ILSI, 2000). The route of exposure, and duration and multiplicity of exposure may also be included in this analysis. The relationship between pathogen dose and illness in host is complex and it may not be possible to fully understand the complete relationship. In some cases the actual dose-response relationship for a pathogen may not be known. In other cases the dose determined (via human feeding studies) using a particular strain may vary when other strains of the same pathogen are used due to strain variability. The disease response will also vary depending on the human. Such things as genetic makeup and immune status can impact a dose-response relationship. Clinical and epidemiological data can be used to generate dose-response curves or models, and when these types of data are not available, animal models may be used. However, interpretations made based on animal models should be done carefully due to differences in host specificity (ILSI, 2000). Once the above data is obtained a host-pathogen profile can be generated. This profile provides a qualitative and/or quantitative description of the nature of the pathogen and potential magnitude of adverse human health affects for the scenario developed during problem formulation. As with the exposure profile, the host-pathogen profile should include limitations and uncertainties that may impact risk characterization (ILSI, 2000).

Risk Characterization Phase. This phase combines the information gathered from the exposure profile and host-pathogen profile and consists of two major elements: risk estimation and risk description. Risk estimation describes the likelihood that a health effect will occur as a result of exposure to a microbial pathogen. It can be expressed as an individual risk estimate (i.e. one person in a million will be affected) or as a
population risk estimate (i.e. 10 illnesses a year). Finally, risk description expresses the confidence in the risk estimates through discussion of weight of evidence (ILSI, 2000). Results from risk characterization are then formulated in language which can inform or communicate the risk to risk managers who use the information to make risk management decisions.

2.6.3.3. Quantitative Microbial Risk Assessment (QMRA) Method Predicts Disease Rate based on Expected Exposure to Water Containing Calculated Concentrations of Pathogens

When epidemiological methods and risk assessment methods cannot be applied to determine the risk for a microbial infection, a relatively new method called quantitative microbial risk assessments (QMRA) can be applied based on direct measurement of pathogens using molecular methods. Some instances when QMRA is advantageous are when epidemiological methods cannot provide sufficient sensitivity to measure risks directly or when the rate of infection is very, very low. When some basic information, such as infectious dose, is known for a pathogen, the QMRA method can be used to calculate risk and to develop guidelines for food, water and other vehicles that may be the source of microbial exposure to human populations (Haas et al., 1999). QMRA can provide an objective and scientific basis for risk management decisions (Medema and Ashbolt, 2006). It can be used to estimate the probability of becoming infected by a specific pathogen after exposure. These types of assessments use known information about densities of the particular pathogen, assumed ingestion rates and dose-response models for the exposed population to estimate the level of risk. Similar to the risk assessment model (section 2.6.3.2), QMRA consists of identifying the pathogen, measuring the duration and intensity of the exposure (exposure assessment) and
analyzing the probability of infection/disease resulting from varying doses of pathogen (dose-response assessment). QMRA can be useful in determining the risk of infection from the use of recreational waters when epidemiological studies are not feasible and when infectious dose can be quantitative to the exposure (e.g., ingestion of pathogens). However, QMRA cannot be applied when the mode of transmission is through inhalation and skin contact because for these modes of transmission, the infectious dose cannot be determined. In summary, QMRA is a theoretical method that determines risk by assessing the hazards and calculating the exposure. QMRA and epidemiological studies can provide complimentary information and should be used together to provide better overall estimates of risk (Pond, 2005).

2.6.3.4. Traditional Methods Cannot be Applied to Assess Risk for Transmission of Pathogenic Vibrio spp. Via Recreational Uses of Coastal Waters

When insufficient data is available for some pathogens, such as Vibrio spp., the approved methods such as epidemiological method, risk assessment method or QMRA method cannot be applied. For these pathogens, the missing information must first be identified and research must be completed to obtain this missing information. A review of the literature shows that the missing information for pathogenic Vibrio spp. in Hawai’i’s coastal waters are as follows: 1) methods to enumerate, speciate and determine the virulence of human pathogenic vibrio bacteria in coastal waters have yet to be approved by USEPA; 2) data is not available to determine the prevalence of human pathogenic Vibrio spp. in various coastal water sites in Hawai’i; 3) temperature, salinity, nutrient levels, and sunlight exposure have been reported to select for different human pathogenic vibrio but the roles of these factors in controlling growth of the various Vibrio spp. in Hawai’i’s coastal waters have not been determined; 4) the expected pathogenicity of
human pathogenic *Vibrio* spp. recovered from coastal waters in Hawai‘i, based on virulence genes, have not been determined; 5) all sources of human pathogenic vibrio bacteria recovered in coastal waters have not been identified. In summary, basic information on pathogenic *Vibrio* spp. must first be obtained for coastal waters in Hawai‘i. Then, based on the information obtained, a modified risk analysis approach will be developed to assess the public health significance of human pathogenic *Vibrio* spp. in coastal waters of Hawai‘i.
CHAPTER 3
THE PROPOSED STUDY

3.1. IDENTIFICATION OF A PROBLEM AND NEEDS FOR A RESEARCH PROJECT

Most of the information about *Vibrio* spp. are based on studies that have been conducted in temperate regions. Information available on the prevalence, sources, and the ecology of these species in tropical areas, such as Hawai‘i, is limited. All four pathogenic *Vibrio* spp. (*V. cholerae*, *V. vulnificus*, *V. parahaemolyticus*, *V. alginolyticus*) have been documented to cause human infections in Hawai‘i. For example, *V. vulnificus* infections have recently resulted in two deaths related to exposure to Hawai‘i’s coastal water sites. One death occurred on the Island of Hawai‘i and the second death occurred on Oahu (Wilson, 2006). Over the past 20 years, four isolated cases of *V. cholerae* infections were confirmed on Oahu in individuals that did not travel outside the state (Leidemann, 2005). The source of *V. cholerae* could not be determined, and contaminated seafood, possibly from coastal waters of Hawai‘i, was considered the most likely source. In addition, infections of *V. parahaemolyticus* were associated with undercooked crabs (Barker, 1974) and surfers with cuts have been infected with *V. alginolyticus* (Blake et al., 1980).

In summary, the identified problem is, human pathogenic *Vibrio* spp. are present in the coastal waters of Hawai‘i and people who use these waters are at risk for infection by these bacteria. However, no studies have been conducted in Hawai‘i to determine whether the four human pathogenic *Vibrio* spp. are found in our coastal waters, especially recreational waters, where exposure is a common daily occurrence. Thus, the identified needs to address this problem are as follows. First, to determine the presence
and the prevalence of the four human pathogenic Vibrio spp. in the different types of recreational coastal waters of Hawai‘i. Second, to assess whether exposure to coastal marine waters represents a risk for infection by human pathogenic vibrios. Third, to determine whether the prevalence data can be used to assess their public health significance and to determine if signage to communicate these risks to the public should be developed for recreational water sites.

3.2. PROJECT GOALS

The first goal of this study was to establish reliable and feasible methods to recover the four human pathogenic Vibrio species (V. cholerae, V. vulnificus, V. parahaemolyticus, V. alginolyticus) from coastal waters of Hawai‘i and to characterize their virulence potential. The second goal was to apply this new method to determine the prevalence of these four human pathogenic vibrio in the major classes, or categories, of coastal waters. The third goal was to determine how selected environmental conditions (salinity, temperature, turbidity, nutrient level) affect the prevalence of the four pathogenic Vibrio spp. in Hawaii’s coastal waters. The fourth goal was to determine if biofilm growth in marine environment or sewage can serve as external sources of Vibrio spp. recovered from water samples obtained from coastal water sites. The fifth goal was to use the prevalence data to assess their public health significance and to determine if signage to communicate these risks to the public should be developed for recreational water sites.
3.3. SELECTION OF THE FOUR CATEGORIES OF COASTAL WATERS

Four categories of coastal waters in Hawai’i were selected based on the following relevant characteristics that have been reported to have predictive impacts on Vibrio spp. concentrations in natural waters: 1) water salinity, 2) water clarity and good exchange with open ocean water, 3) water temperature, 4) expected pollution of coastal waters by land-based runoff, 5) designation as sites approved or not approved for swimming, 6) sites identified as previous site of transmission of vibrio infection.

Category 1. Primary Coastal Beaches: Approved as best swimming beaches on Oahu. Water at these beaches are characterized by consistently high salinity, low turbidity, good exchange of water with open ocean, and these sites are not impacted by nearby land-based runoff.

Category 2. Secondary Coastal Beaches: Approved swimming beaches on Oahu with variable salinity and turbidity due to variable contamination by nearby land-based runoff.

Category 3. Coastal Harbors, Ponds and Canals on Oahu: Not approved for swimming because these sites are consistently and heavily impacted by pollution from land-based runoff. These sites are confined coastal bodies that have poor circulation with the open ocean and receive discharges from land-based discharges such as streams and storm drains. Water at these sites have variable salinities and variable to elevated turbidity. Although these sites are not approved for swimming, recreational activities occur at these sites by some people. A case of accidental contact with water from Ala Wai Canal resulted in infection and death caused by V. vulnificus.
Category 4. Coastal swimming sites on the Island of Hawai‘i: These sites are approved for swimming and have a history of transmitting vibrio infections. These coastal water sites are not typical swimming beaches as compared to beaches on Oahu. The reason is the age of the island of Hawai‘i (< 1 million years old) which is much younger than the island of Oahu (2-3 million years old). As a result, the hydrogeology of this island is characterized by cooled lava formation and differs from that on the island of Oahu where lava formation has degraded to more typical soil and subsoil formations. Thus, the sandy beaches observed on the island of Oahu have not developed on the island of Hawai‘i. Moreover, due to the predominating subterranean lava formation in coastal areas of this island, shallow groundwater discharges occur at many coastal sites in the absence of obvious land based discharges such as streams or storm drains. In this regard, groundwater near the Puna district discharge volcano heated water forming low salinity, thermal pond. Transmission of *V. vulnificus* infection was reported from one of the thermal ponds in this area. On the Kona side of the island, cool groundwater discharge into coastal water sites, which often form ponds close to shore, are conducive for swimming. Transmission of vibrio type skin infections have also been reported from these ponds in the Kona area. Water at coastal sites on the island of Hawai‘i can be characterized as having variable salinity, turbidity and water temperature.

3.4. EXPERIMENTAL DESIGN TO ASSESS THE PUBLIC HEALTH SIGNIFICANCE OF PATHOGENIC *VIBRIO* SPP. IN HAWAII’S COASTAL WATERS

As stated in Section 2.6.3, epidemiological methods to develop water quality standards or the standard risk assessment method or the risk analysis QMRA method cannot be applied to interpret data collected related to pathogenic *Vibrio* spp. in the
coastal waters of Hawai‘i. As a result, a new experimental approach will need to be
developed to interpret the water monitoring data for pathogenic *Vibrio* spp. In developing
this new approach, we are aware that the USEPA epidemiological study and current
recreational water quality standards, based on concentrations of fecal indicator bacteria,
are not reliable in Hawai‘i. The basic problem has been in effectively communicating the
risk to the public when water quality standards are exceeded. We are also aware that in
Hawai‘i, we have obtained water monitoring data for *Staphylococcus aureus* in beach
waters, however, the problem has been the inability to set water quality standards for *S.
aureus* and how best to communicate the risk to the public based on monitoring data. In
Hawai‘i, we are also aware that the State has taken a public health strategy of placing
signs at all freshwater stream and storm drain sites warning of the risk for infection with
*Leptospira* sp. when exposed to freshwater environments. This too is not a satisfactory
policy as there is no information to inform the public as to the relative risk of using
different freshwater sites in Hawai‘i.

For this study, the monitoring data for pathogenic *Vibrio* spp. will use prevalence
data, for each of the four pathogenic species of *Vibrio*, as the potential source of
infection. The severity of illness for each of the pathogenic *Vibrio* spp. will be
determined based on epidemiological data gathered by the CDC between 1997 and 2008,
and will be determined based on the number of infections and deaths attributed to each
species. Based on the prevalence data gathered from this study and the determined
severity of illness the public health significance for each *Vibrio* spp. will be determined at
each of the four water categories. Finally this public health significance information will
be used to develop a risk communication method to inform the public where the risks for vibrio infection are the greatest.

3.5. SAMPLING SITES AND METHODS FOR COLLECTION

The location of the four categories of water sites on the islands of Oahu and Hawai‘i are shown in Appendix A. One-liter water samples were collected into sterile plastic sampling bottles at waist depth from all primary and secondary swimming beaches. For harbor, canal and pond sites, a clean bucket was used to collect water samples which were then transferred into sterile, one-liter sampling bottles. Sediment samples were taken at knee depth using a strainer containing a nylon mesh. Three sediment samples were taken from each site, pooled into sterile Whirl-Pak bags and transported to the laboratory for analysis. All samples were analyzed within three hours of collection. Water samples from the Island of Hawai‘i were collected into sterile, plastic, one-liter sampling bottles and transported to the laboratory for analysis. Due to transport times, these samples were processed within 8 hours of collection. Wastewater (sewage) samples were collected in sterile, 500-ml, plastic bottles and transported to the laboratory. Samples were analyzed within 3 hours of collection. All samples were transported to the laboratory in a cooler (without ice) to keep the samples at ambient temperature and protected from sunlight exposure.

3.6. PHYSICAL WATER QUALITY CHARACTERISTICS (TURBIDITY AND SALINITY)

Turbidity and salinity measurements were taken of water samples. Turbidity was measured using a Hach Turbidometer (model 2100N) and units were recorded as NTU (Nephelometric Turbidity Units). Salinity was measured using a refractometer
(Aquafauna model) and recorded as parts per thousand (ppt). Both turbidity and salinity measurements were used to determine whether sites were impacted by freshwater/groundwater discharges.

3.7. CULTURE METHODS TO ASSAY FOR VIBRIO SPP.

3.7.1. Water and Sewage Samples

The membrane filtration procedure was used for the enumeration of total marine bacteria and vibrio bacteria. A peptone buffer (PB) solution containing 0.1% peptone and 3% NaCl (Azanza et al., 1996) was used to dilute samples prior to filtration. Water samples were diluted as needed into sterile PB, and 25ml portions were filtered through a 0.45 μm pore size filter (Gelman GN-6). These filters were then placed onto either marine agar (MA), thiosulfate citrate bile salts sucrose (TCBS) agar or CHROMagar Vibrio (CV). All media were prepared in accordance to manufacturer specifications. Plates were incubated for 24 hours at 35°C. After the incubation period, colonies on each plate were counted. Approximately 20% of turquoise colonies, 20% of mauve colonies and 10% of colorless colonies were picked off CV plates using sterile toothpicks and streaked for isolation on fresh CV plates. Isolated colonies were then streaked onto tryptic soy agar + 0.5% NaCl (TSA+0.5% NaCl) plates for biochemical testing. Figure 3.1 is a schematic representation of the method used to process samples.

3.7.2. Sediment Samples

Bacteria were eluted from sediment samples using a modified procedure used by the United States Geological Survey (USGS) (Myers et al., 2003). Samples (100 g) were placed into a sterile, 500-ml plastic bottle and mixed well using a sterile, wooden spatula. Then, 200 ml of PB was added to the bottle and the bottle was shaken vigorously by hand
for 5 minutes. Upon shaking, the sediment sample was allowed to settle for 30 seconds
and the eluate was collected into a separate sterile bottle. The elution process was done
twice for each sediment sample and the two supernatants were combined. A double

Figure 3.1. Schematic Diagram of the Processing of Water Samples and Characterization
of Vibrio Isolates.

elution was used because in preliminary experiments, it was shown that this process was
capable of eluting approximately 98% of bacteria attached to sediment particles. The
pooled eluates were processed in a similar manner as water samples. Colonies of bacteria
from CV plates were collected using sterile toothpicks for further analyses. To determine
the dry weight of the sediment sample, a small aliquot (10 g) of each sediment sample
was placed onto a drying dish and dried in a 105°C oven for 24 hours.
3.8. METHODS USED TO IDENTIFY AND SPECIATE THE VIBRIO ISOLATES

3.8.1. Presumptive Identification Using USFDA Testing Procedure

In order to make a presumptive identification of isolates picked off CV plates, two biochemical test procedures (sucrose fermentation, growth in various concentrations of salt) as described in USFDA Bacteriological Analytical Manual (BAM), were used to speciate the vibrio isolates (DePaola and Kaysner, 2004). A 24 hour culture of each isolate grown on TSA+0.5% NaCl was used for biochemical testing. Sucrose fermentation was conducted on TCBS agar. Isolates from TSA+0.5% NaCl plates were streaked onto TCBS plates using sterile toothpicks and the plates were incubated for 24 hours at 35°C. After the incubation period, isolates were scored as sucrose positive or sucrose negative. Isolates were also subjected to salt tolerance tests. Turquoise, mauve and colorless colonies were tested for growth at four salt concentrations (0%, 6%, 8%, 10%). Salt broths were made by adding appropriate amounts of NaCl to nutrient broth (NB) as described by the USFDA BAM. Briefly, no NaCl was added to the 0% broth, 6 g of NaCl per 100 ml of NB was added for the 6% broth, 8 g of NaCl per 100 ml of NB was added for the 8% broth and 10g of NaCl per 100 ml of NB was added for the 10% broth. Salt broths were placed onto a stir plate with a stir bar and mixed until the NaCl had dissolved completely. After mixing, 5 ml of each broth was transferred into glass screw cap tubes and sterilized by autoclaving. A 2% saline broth (2 g NaCl per 100 ml distilled water) was also made, and 1 ml was transferred into screw cap tubes and sterilized by autoclaving. Prior to inoculating the salt tubes, a single colony of each isolate was picked off the TSA+0.5% NaCl plate and homogenized into a tube of 2% saline broth. 0.1 ml of the saline broth tube was then used to inoculate each of the salt
tubes (0%, 6%, 8%, 10%). The tubes were incubated at 35°C for seven days and scored as growth being present or absent (Choopun et al., 2002). Based on sucrose fermentation and salt tolerance, isolates were presumptively identified as *V. cholerae*, *V. vulnificus*, *V. parahaemolyticus* or *V. alginolyticus* and then confirmed by species specific PCR.

### 3.8.2. Extraction of *V. cholerae*, *V. vulnificus* and *V. parahaemolyticus* DNA

DNA of presumptive vibrio isolates was extracted according to a method described by Lee et al (2004). Briefly, isolates were grown overnight at 35°C in 1 ml of tryptose soy broth containing 1.5% NaCl. The culture was then boiled at 100°C on a heat block for 5 minutes and centrifuged for 5 minutes at 10,000 RPM in a micro-centrifuge. The supernatant was then directly used for PCR reactions. Species specific PCR primers for *V. cholerae*, *V. vulnificus* and *V. parahaemolyticus* were used to confirm presumptive isolates from all samples. All primers were purchased from the Greenwood Molecular Biology Facility located at the University of Hawai`i at Mānoa.

### 3.8.3. Confirmation of Presumptive Isolates Using Species Specific PCR Primers

Table 3.1 is a list of the specific primers that were used for each species. Presumptive *V. cholerae* isolates were confirmed by primers developed by Chun et al (1999). These primers target the intergenic spacer region (ISR) between the 16S and 23S rDNA. These regions are thought to be under less evolutionary pressure than rRNA coding regions and can be used to differentiate between closely related organisms. These primers were shown to be able to differentiate between the two closely related species *V. cholerae* and *V. mimicus*. The protocol for PCR amplification described by Chun et al
(1999) was followed and the resulting PCR product was visualized on a 1.5% agarose gel.

Table 3.1. List of gene targets and references of PCR primers used for confirmation of *V. cholerae*, *V. vulnificus* and *V. parahaemolyticus* isolates.

<table>
<thead>
<tr>
<th>Species</th>
<th>Target Gene</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>V. cholerae</em></td>
<td>16S-23S rDNA ISR</td>
<td>Chun et al 1999</td>
</tr>
<tr>
<td><em>V. vulnificus</em></td>
<td>16S rDNA</td>
<td>Kim and Jeong 2001</td>
</tr>
<tr>
<td><em>V. parahaemolyticus</em></td>
<td>pR72H</td>
<td>Lee et al 1995</td>
</tr>
</tbody>
</table>

For confirmation of presumptive *V. vulnificus* isolates primers developed by Kim and Jeong (2001) were used. By analysis of known 16S rRNA sequences of *Vibrio* species, four variable regions were identified that were used as target sequences for primers specific for the amplification of 16S rRNA genes of *V. vulnificus*. 16S rRNA genes are essential constituents of all living organisms. Thus, there is no problem with gene loss which may be a possibility with hemolysin/cytolysin genes. In addition, 16S rRNA genes are present in high copy numbers within cells. The protocol for PCR amplification described by Kim and Jeong was followed, and the resulting PCR product was visualized on a 1.5% agarose gel.

PCR confirmation of presumptive *V. parahaemolyticus* isolates relied on primers developed by Lee et al (1995). Because environmental strains of *V. parahaemolyticus* generally do not produce hemolysins as clinical strains do, the use of markers for hemolysin genes may not be useful. Thus, in this study, primers developed by Lee et al (1995) that target a fragment on the chromosomal DNA of *V. parahaemolyticus* named pR72H was used. The function of this region of DNA is unknown but was shown to be conserved among *V. parahaemolyticus* strains. The protocol for PCR amplification
described by Lee et al (1995) was followed, and the resulting PCR product was visualized on a 1.5% agarose gel.

3.9. METHODS USED TO ASSAY FOR VIRULENCE DETERMINANTS

Isolates of *V. cholerae*, *V. vulnificus* and *V. parahaemolyticus* that were positively confirmed using species specific primers were then subjected to PCR conditions to determine the presence of virulence determinants. Table 3.2 lists the various virulence determinants of each species that were targeted, as well as the corresponding reference.

For *V. cholerae* isolates, primers that targeted the presence of genes of subunit A of cholera toxin and genes specific for the two pandemic serotypes of this strain were used. DNA was extracted in a similar manner as described above and the protocol for PCR amplification described by Lipp et al (2003) was followed for PCR amplification. The resulting PCR product was visualized on a 1.5% agarose gel.

Table 3.2. List of virulence determinants and references of PCR primers used for *V. cholerae*, *V. vulnificus* and *V. parahaemolyticus* isolates.

<table>
<thead>
<tr>
<th>Species</th>
<th>Virulence Determinant</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>V. cholerae</em></td>
<td>ctxA</td>
<td>Lipp et al 2003</td>
</tr>
<tr>
<td></td>
<td>01</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0139</td>
<td></td>
</tr>
<tr>
<td><em>V. vulnificus</em></td>
<td>A/B Subtypes</td>
<td>Kim and Jeong 2001</td>
</tr>
<tr>
<td><em>V. parahaemolyticus</em></td>
<td>tdh</td>
<td>Bej et al 1999</td>
</tr>
<tr>
<td></td>
<td>trh</td>
<td></td>
</tr>
</tbody>
</table>

*V. vulnificus* isolates that were positively confirmed by species specific PCR were subtyped using a method described by Kim and Jeong (2001). Essentially, this method is able to type this species into either environmental (less virulent) or clinical strains (more virulent) based on two different 16S rRNA sequences: type A (environmental) or type B (clinical). DNA was extracted in a similar manner as described above and the protocol
for PCR amplification described by Kim and Jeong (2001) was followed for PCR amplification. The resulting PCR product was visualized on a 1.5% agarose gel.

Positively identified *V. parahaemolyticus* isolates were subjected to PCR conditions to determine the presence of genes associated with two hemolysins: TDH and TRH. DNA was extracted in a similar manner as described above and the protocols for PCR amplification for detection of *tdh* and *trh* were followed as described by Bej et al (1999). The resulting PCR product was visualized on a 1.5% agarose gel.

3.10. EXPERIMENTAL DESIGN TO USE A PREVALENCE AND SEVERITY INDEX TO DETERMINE RELATIVE PUBLIC HEALTH SIGNIFICANCE OF PATHOGENIC *VIBRIO* SPP. IN HAWAI'I'S COASTAL WATERS

3.10.1. Prevalence Index: Usefulness and Limitations

Although, methods to directly enumerate *V. vulnificus*, *V. cholerae*, *V. parahaemolyticus* and *V. alginolyticus* are not available, we developed a method of determining the percent of samples, or prevalence index, for these four human pathogenic *Vibrio* spp. in the four categories of coastal waters in Hawai‘i. The experimental design to determine the public health significance for these four pathogenic *Vibrio* spp. was to initially determine the prevalence index as a probability index for causing an infection when people are exposed to coastal waters. Since, prevalence data was equated with probability for infection, the following guidelines for probability for infection were used: 1) water sites that average 90% prevalence for a given *Vibrio* sp. was given a very high probability for infection; 2) water sites that average 75% prevalence for a given *Vibrio* sp. was given a high probability of infection; 3) water sites that average 50% prevalence for a given *Vibrio* sp. was given a moderate probability for infection; 4) water sites that average 25% prevalence for a given *Vibrio* sp. was given a low probability for infection;
5) water sites that averaged 5% prevalence for a given *Vibrio* sp. was given a very low probability for infection and 6) water sites in which the *Vibrio* sp. could not be detected was given an undetectable probability for infection. These prevalence indices are summarized in Table 3.3.

Table 3.3. The range of percent recovery for each *Vibrio* spp. are used to determine the prevalence index for each *Vibrio* spp.

<table>
<thead>
<tr>
<th>Prevalence Range of <em>Vibrio</em> Species (%)</th>
<th>Prevalence Index</th>
</tr>
</thead>
<tbody>
<tr>
<td>90-100</td>
<td>Very High</td>
</tr>
<tr>
<td>70-90</td>
<td>High</td>
</tr>
<tr>
<td>30-70</td>
<td>Moderate</td>
</tr>
<tr>
<td>10-30</td>
<td>Low</td>
</tr>
<tr>
<td>1-10</td>
<td>Very Low</td>
</tr>
<tr>
<td>&lt;1</td>
<td>Undetectable</td>
</tr>
</tbody>
</table>

This prevalence index, or the percent of samples positive for each of the four pathogenic *Vibrio* spp., can be used to determine the relative probability for infection of people exposed to that water. However, it must be pointed out that the prevalence data cannot be used to determine risk of infection because the concentrations and virulence capacity of each of the pathogenic vibrio recovered from that coastal water samples cannot be enumerated. Since a prevalence index for the four pathogenic *Vibrio* spp. has not been previously done, but can be theoretically accomplished, the experimental design of this study was to determine the prevalence index for the four human pathogenic *Vibrio* spp. in the four categories of coastal water environment in Hawai‘i.

3.10.2. Severity Index: Usefulness and Limitations

The probability of infection is only the first level of concern. The second level of concern is the severity index or the severity of the disease symptoms when infections by
these four *Vibrio* spp. develop into their characteristic disease symptoms. The disease symptoms that result in the likelihood of death (mortality index) were classified as high, medium or low. Disease symptoms that do not usually develop in death (mortality) but can result in morbidity symptoms or incapacitating symptoms were classified as having a high morbidity index. The disease symptoms that are characterized with negligible or low mortality and low morbidity index would be classified as having a low morbidity index.

A relative severity index based on one published by Pond (2005) was used in this study. Establishment of this index was limited to the data that was applicable to environmental waters in the U.S. and under the environmental and sanitary conditions, and health status for the people in the U.S. Table 3.4 uses published epidemiological data gathered by the CDC between 1997 and 2008 (CDC, 2010a) to determine the severity index for each of the four *Vibrio* spp. The severity takes into account the relative number of infections and the number of deaths attributed to each species during this time period. The severity index was scored “High” if numbers of both infections and deaths attributed to the species were high while a score of “Low” was give if numbers of both infections and deaths were low. One limitation of using this CDC data to determine the relative severity of each *Vibrio* spp. is that the data includes the number of infections and deaths related to both food consumption as well as wound infections obtained from non-recreational water uses. Although the disease symptoms are similar, the data directly related to recreational uses of water remains low. However, wound associated infections and deaths following recreational exposure to coastal waters follow a similar trend in severity (i.e., *V. vulnificus* causes the most severe wound infections). It should be noted that, historically, *V. cholerae* infections have caused severe infections and high mortality.
rates. However, infections and deaths related to this species is generally low in industrialized countries, as the U.S, which contributes to its low severity score.

Table 3.4. The relative severity of illness index for *V. vulnificus*, *V. parahaemolyticus*, *V. cholerae* and *V. alginolyticus* based on epidemiological data gathered by the CDC in Gulf Coast States from 1997-2008.

<table>
<thead>
<tr>
<th><em>Vibrio</em> Species</th>
<th>No. Infections&lt;sup&gt;a&lt;/sup&gt;</th>
<th>No. Deaths&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Severity Index</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>V. vulnificus</em></td>
<td>High</td>
<td>High</td>
<td>High</td>
</tr>
<tr>
<td><em>V. parahaemolyticus</em></td>
<td>High</td>
<td>Low</td>
<td>Moderate</td>
</tr>
<tr>
<td><em>V. cholerae</em></td>
<td>Very Low</td>
<td>Very Low</td>
<td>Very Low</td>
</tr>
<tr>
<td><em>V. alginolyticus</em></td>
<td>Low</td>
<td>Very Low</td>
<td>Low</td>
</tr>
</tbody>
</table>

<sup>a</sup> the relative number of infections attributed to the species from 1997-2008.

<sup>b</sup> the relative number of deaths associated with the species from 1997-2008.

3.10.3. Evaluation of Infectious Dose for Ingestion versus Dermal Contact Transmission

When a person is exposed to coastal waters containing pathogens, the first issue is the probability of infection. There are many factors that control whether the pathogen in the water will infect the person exposed to that water. Important factors include the susceptibility of the host, mode of infection (ingestion, dermal), the required concentration of the pathogen needed to cause infection (infectious dose) as well as the virulence of the pathogen. Laboratory feeding studies have clearly shown that the minimal infectious ingestion dose of virulent strains of *V. vulnificus* or *V. cholerae* or *V. parahaemolyticus*, for an average person, is very high (10<sup>6</sup> –10<sup>11</sup> live cells). Filter feeding organisms (oyster, clams) are known to concentrate and harbor large numbers of vibrio bacteria from surrounding waters in their tissue. When these oysters are eaten uncooked or inadequately cooked by an individual, the concentrations of pathogenic *V. vulnificus* and *V. parahaemolyticus* have often resulted in mild to severe gastrointestinal illness and even death. Thus, ingestion of seafood remains the most common route of
infection for *V. vulnificus* and *V. parahaemolyticus* in the U.S. The likelihood that gastrointestinal illness will result from exposure to recreational waters is minimal because the concentrations of pathogenic *Vibrio* spp. is not expected to reach infectious dose levels. As a result, individuals who swim and recreate in these waters are not expected to ingest the high levels (10^6 cells) of bacteria needed to cause disease. Rather, contact with recreational waters is more likely to lead to dermal infections, either as a result of infection of a preexisting wound or a wound acquired during recreational activities. Currently the infectious dose required to cause dermal infections for *Vibrio* spp. is not known. Thus, it may be possible for even a small number of bacterial cells to cause dermal infections once these bacteria enter the broken skin. In addition, individuals who spend extended periods of time in contact with coastal waters are more likely to come into contact with aggregates of vibrio bacteria and may have a higher chance of contracting dermal infections. It should also be noted that *V. vulnificus* infections have resulted from wounds as small as an ant bite (Oliver, 2006), making it likely that individuals may not even be aware they have open sites for infection. Furthermore, it is known that the minimal infectious dose can be greatly reduced when the person or host becomes highly susceptible due to liver diseases, compromised immunity, or when the acidity of the stomach decreases (pH increases) due to the use of antacid pills. Very little is known about infectious doses for dermal infections, however, some of the same host factors that make people more susceptible to vibrio infections are expected to lower the infectious dose for these types infections.
3.10.4. Use of Risk Communication to Assess Public Health Significance of Four Human Pathogenic *Vibrio* spp. in Coastal Waters

Currently insufficient data is available to predict the probability of infection when human pathogenic vibrio are present in coastal waters. Some of the missing information for pathogenic vibrio are as follows: 1) *Vibrio* spp. are natural marine bacteria and conditions or factors in the marine environment that control the concentrations of each of the four pathogenic *Vibrio* spp. are not known; 2) methods to selectively measure the concentrations of the four pathogenic *Vibrio* spp. are not available; 3) data to document the concentrations of the four pathogenic *Vibrio* spp. in coastal water environments of Hawai`i have not been established; 4) there are no known major terrestrial sources of pathogenic *Vibrio* spp. which can be treated to reduce the concentrations in coastal waters; 5) the same pathogenic *Vibrio* spp. can infect humans via ingestion or by penetrating the skin of people, and, 6) the infectious doses for infecting humans who are exposed to recreational waters is not known for the dermal contact route.

As previously stated, current USEPA water quality standards for fecal pollution are based on epidemiological studies conducted in temperate areas of the U.S. then applied to all areas of the U.S. (temperate and tropical). These standards however have been shown to be unreliable in Hawai`i because, in tropical climates, the fecal indicator microorganisms (*E. coli*, enterococci) are known to be naturally found in the environment (i.e., soils). Thus, the epidemiological studies conducted in temperate U.S., and the resulting standards, have failed to accurately assess risk to users of Hawai`i’s recreational waters. A second risk management strategy that has failed in Hawai`i involves the Hawaii Department of Health (HDOH) approach to prevent infection by *Leptospira* spp. which are present in the fresh water streams of Hawai`i. Since there are no methods to
measure for pathogenic *Leptospira* spp. in stream water samples, HDOH uses a risk communication approach of posting warning signs at all stream sites in the state. The warning sign alert the populace that leptospira bacteria may be in stream waters and can infect swimmers and bathers, especially those with open wounds. This is an unsatisfactory strategy because it is not based on a measured standard and assumes the same unacceptable risk at all streams in Hawai’i.

Beach managers know that infection without disease is a public health consequence that does not rise to the level for a plan of action. However, these managers are aware that infections that can lead to serious consequences, such as death and secondary transmission, must be taken seriously. For infections with these kinds of probability, an active management or mitigation plan of action must be developed before the overt and serious disease symptoms are observed. Because of the gaps in information present in relation to *Vibrio* spp., this study used a prevalence index and a severity index to determine the public health significance for each *Vibrio* spp. and to develop a risk communication approach for the purpose of determining the need for posting a warning sign and the nature of the wording of the posted warning sign at the four categories of coastal waters.

### 3.10.4.1. Public Health Significance for *V. cholerae* Disease Transmitted via Recreational Uses of Coastal Water

Cholera infections are generally acquired from drinking water that has been contaminated by the feces of a symptomatically or asymptptomatically infected person or through contaminated seafood. Because the infectious dose needed to cause gastrointestinal illness is large (>10^6 cells), it is not likely that infection will result from
ingesting recreational waters. Moreover, *V. cholerae* is known to grow in low salinity but not in high salinity waters found in most coastal swimming beaches. Although wound infections due to *V. cholerae* have been known to occur after wound exposure to contaminated waters, these types of infections are generally rare and non-fatal. Because the severity index for this species is considered very low in the U.S., the risk for infection by *V. cholerae* related to exposure at coastal swimming water sites in Hawai‘i is considered unlikely and therefore to be of low public health significance. Based on this assessment, there is no need to post warning signage for *V. cholerae* infection at coastal water sites in Hawai‘i.

3.10.4.2. Public Health Significance for *V. alginolyticus* Disease Transmitted via Recreational Uses of Coastal Water

Though *V. alginolyticus* is generally the predominant *Vibrio* species in many parts of the world, infections are generally mild and self limiting, lasting only one to two days. Necrotizing fasciitis can occur but is generally seen in individuals with underlying condition such as cirrhosis. Isolation of *V. alginolyticus* from cases of diarrhea or gastroenteritis is rare and exposure to seawater is considered the primary risk factor for infection by this species. Normal swimming activities are not considered a risk factor for *V. alginolyticus* infection, however, certain recreational activities, such as surfing or diving, can put individuals at risk for infection by this species. This is because engaging in these activities can result in coral cuts or skin abrasions which can then become infected by this *Vibrio* sp. Because the severity index for *V. alginolyticus* is considered low, the likelihood that this *Vibrio* spp. will result in transmitting diseases is considered
to be of low public health significance. Based on this assessment, there is no need to post warning signage for *V. alginolyticus* infection at coastal water sites in Hawai‘i.

### 3.10.4.3. Public Health Significance for *V. parahaemolyticus* Disease Transmitted via Recreational Uses of Coastal Water

*V. parahaemolyticus* can cause three types of clinical disease: gastroenteritis, wound infections and septicemia, but is primarily a foodborne illness. Gastrointestinal symptoms are generally self-limiting and resolve within a few days. Though less common, *V. parahaemolyticus* can also cause an infection of the skin when an open wound is exposed to warm seawater. These types of infections are usually mild but can be severe in individuals with underlying health conditions (diabetes, liver disease). Genes for two hemolysins have been used as markers to distinguish virulent strains of *V. parahaemolyticus* from non-virulent ones. Strains isolated from diarrheal patients have been shown to produce either the thermostable direct hemolysin (TDH), TDH-related hemolysin (TRH) or both, while environmental strains are generally incapable of producing these hemolysins. Though the severity index for *V. parahaemolyticus* is considered moderate, the likelihood that this *Vibrio* spp. will result in transmitting diseases due to exposure to coastal waters is considered to be of low public health significance. Based on this assessment, there is no need to post warning signage for *V. parahaemolyticus* infection at coastal water sites in Hawai‘i.

### 3.10.4.4. Public Health Significance for *V. vulnificus* Disease Transmitted via Recreational Uses of Coastal Water

*V. vulnificus* is found in temperate and tropical waters around the world and can be isolated from seawater, sediment, and various marine life forms, particularly when
water temperatures are warm (summer months in temperate regions, year round in tropical regions). It can cause three types of disease: gastroenteritis, wound infections and septicemia, and is the only *Vibrio* spp. that is capable of causing severe illness that is transmitted by exposure to coastal water. Although gastroenteritis is self-limiting and rarely reported, wound infections and primary septicemia are highly lethal conditions that occur most often among persons with underlying conditions (liver disease, immunocompromised) which make them highly susceptible to infection and disease by this species. Wound infections that become necrotic may require amputation, and in certain individuals may result in death. Approximately 95% of people who die from wound infections have some underlying health symptoms. However, some people with no measurable underlying conditions have also succumbed to *V. vulnificus* infection. Symptoms of *V. vulnificus* wound infections develop rapidly and death can occur within a few days. Because the severity index for *V. vulnificus* is considered high, and because this *Vibrio* sp. is known to be present in coastal waters of the U.S., the public health significance of this vibrio in coastal is considered to be of high public health significance. Based on this assessment, there is a need to post warning signage for *V. vulnificus* infection at coastal water sites in Hawai`i where the prevalence of this pathogenic vibrio has been documented.
CHAPTER 4

ASSESSING THE PREVALENCE OF PATHOGENIC *VIBRIO*
SPECIES IN THE FOUR CATEGORIES OF COASTAL WATERS
ON THE ISLANDS OF OAHU AND HAWAII

4.1. INTRODUCTION

Much of what we know about the prevalence and ecology of pathogenic *Vibrio* species are based on studies conducted in temperate areas of the world. Similar data is limited in tropical areas such as Hawaii where no seasonal climate change occurs. These changes can have an effect on the distribution of microbial pathogens, particularly pathogenic *Vibrio* species that have been shown to be affected in large part by water temperature and salinity variations. In tropical areas, such as Hawaii, near-shore coastal water temperatures are relatively constant throughout the year, thus it would be expected that salinity may play a more significant role in the prevalence of pathogenic *Vibrio* species in these regions. In addition, the changing global climate is expected to have an effect on these and other pathogens (Colwell, 1996; Lobitz et al., 2000; Lipp et al., 2002). Two papers independently reported that warming in the top 3,000 meters of the global ocean had occurred since 1950 (Barnett et al., 2001; Levitus et al., 2001). In relation to *Vibrio* spp. abundance, one study has shown that increasing sea water temperatures have contributed to an increase in coral bleaching by pathogenic *Vibrio* species in the Mediterranean Sea (Vezzulli et al., 2010). This same study has experimentally shown that these coral pathogens are more virulent when water temperatures are warmer. A second study described the increased incidence of *V. cholerae* along the coast of Peru due to climate associated increases in water temperature (Gil et al., 2004). If the trend of warming oceans continues, we can expect to see increased prevalence and illness due to contact with pathogenic *Vibrio* species.
Tropical regions such as Hawaii are known for their warm waters year-round. Thus, unlike in temperate areas where the culturability of *Vibrio* species decreases during the winter months (Motes, 1998), these species should be recoverable in Hawaii waters throughout the year. In addition, Hawaii is a popular destination among visitors who come to recreate in our coastal waters. What is not known is the actual risk for infection with *Vibrio* spp. for people who swim in coastal waters of Hawaii.

### 4.2. STUDY OBJECTIVES

#### 4.2.1. Objective 1:
To evaluate and determine the most feasible method to recover, enumerate and identify the four human pathogenic *Vibrio* spp. from the selected categories of coastal waters.

#### 4.2.2. Objective 2:
To determine the prevalence of the four human pathogenic *Vibrio* spp. in the following four selected categories of coastal waters:

**Category 1. Primary Coastal Beaches:** Approved as best swimming beaches on Oahu. Water at these beaches are characterized by consistently high salinity, low turbidity, good exchange of water with open ocean, and these sites are not impacted by nearby land-based runoff.

**Category 2. Secondary Coastal Beaches:** Approved swimming beaches on Oahu with variable salinity and turbidity due to variable contamination by nearby land-based runoff.

**Category 3. Coastal Harbors, Ponds and Canals on Oahu:** Not approved for swimming because these sites are consistently and heavily impacted by pollution from land-based runoff. These sites are confined coastal bodies that have poor
circulation with the open ocean and receive discharges from land-based
discharges such as streams and storm drains. Water at these sites have variable
salinities and variable to elevated turbidity. Although these sites are not approved
for swimming, recreational activities occur at these sites by some people. A case
of accidental contact with water from Ala Wai Canal resulted in infection and
death caused by *V. vulnificus*.

**Category 4. Coastal swimming sites on the Island of Hawai‘i:** These sites are
approved for swimming and have a history of transmitting vibrio infections.
These coastal water sites are not typical swimming beaches as compared to
beaches on Oahu. The reason is the age of the island of Hawai‘i (< 1 million
years old) which is much younger than the island of Oahu (2-3 million years old).
As a result, the hydrogeology of this island is characterized by cooled lava
formation and differs from that on the island of Oahu where lava formation has
degraded to more typical soil and subsoil formations. Thus, the sandy beaches
observed on the island of Oahu have not developed on the island of Hawai‘i.
Moreover, due to the predominating subterranean lava formation in coastal areas
of this island, shallow groundwater discharges occur at many coastal sites in the
absence of obvious land based discharges such as streams or storm drains. In this
regard, groundwater near the Puna district discharge volcano heated water
forming low salinity, thermal ponds. Transmission of *V. vulnificus* infection was
reported from one of the thermal ponds in this area. On the Kona side of the
island, cool groundwater discharge into coastal water sites, which often form
ponds close to shore, are conducive for swimming. Transmission of vibrio type
skin infections have also been reported from these ponds in the Kona area. Water at coastal sites on the island of Hawai‘i can be characterized as having variable salinity, turbidity and water temperature.

**4.2.3. Objective 3:** To determine whether environmental conditions that had been previously reported to control growth of the four human pathogenic *Vibrio* spp. can be used to explain the prevalence data of the four *Vibrio* spp. recovered from the four selected categories of coastal waters.

**4.2.4. Hypotheses.** The following are some stated hypotheses which are used to predict the prevalence of the four pathogenic *Vibrio* spp. in the various coastal waters of Hawai‘i:

**4.2.4.1. Hypothesis 1:** Primary Coastal Beaches on Oahu. The high salinity (35 ppt), and low nutrients will select for *V. alginolyticus*, which is known to prefer high salinity for growth.

**4.2.4.2. Hypothesis 2:** Secondary Coastal Beaches on Oahu. The variable salinity and variable nutrient levels will select for *V. parahaemolyticus*, *V. vulnificus* and *V. alginolyticus* because the salinity range and nutrient levels at this site are favorable for these three *Vibrio* spp.

**4.2.4.3. Hypothesis 3:** Coastal Harbors, Ponds and Canals on Oahu. The variable salinity and variable nutrient levels will select for *V. parahaemolyticus*, *V. vulnificus* and *V. alginolyticus*, because the salinity range (12-34 ppt) and nutrient level at this site are favorable for these three *Vibrio* spp.
4.2.4.4. **Hypothesis 4:** Coastal ponds on the Island of Hawai‘i. The variable salinity and variable nutrient level will select for *V. parahaemolyticus*, *V. vulnificus*, *V. alginolyticus* and *V. cholerae* because the salinity range (9-21 ppt) and nutrient levels at this site are favorable for these four *Vibrio* spp.

4.3. **SAMPLING SITES AND EXPERIMENTAL DESIGN**

Sampling sites on the islands of Oahu and Hawaii were categorized into four groups based on their water quality characteristics. The four water quality categories are Table 4.1. Description of the four water quality categories that were sampled on the Islands of Oahu and Hawaii.

<table>
<thead>
<tr>
<th>Designated Categories Of Coastal Waters</th>
<th>No. of Sites Sampled (n)</th>
<th>Description of Quality of Water</th>
<th>Approved for Swimming?</th>
<th>Land Run-off Present?</th>
</tr>
</thead>
<tbody>
<tr>
<td>I. Primary Coastal Beaches on Oahu</td>
<td>20</td>
<td></td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>II. Secondary Coastal Beaches on Oahu</td>
<td>15</td>
<td></td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>III. Coastal Harbors, Canals and Ponds on Oahu</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A. Harbors</td>
<td>11</td>
<td></td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>B. Canals</td>
<td>7</td>
<td></td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>C. Ponds</td>
<td>5</td>
<td></td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>IV. Coastal Swimming Sites on Hawaii</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A. Kona Area</td>
<td>9</td>
<td></td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>B. Hilo Area</td>
<td>8</td>
<td></td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>C. High Temp., Low Salinity Ponds on Island of Hawaii</td>
<td>4</td>
<td></td>
<td>Yes</td>
<td>Yes</td>
</tr>
</tbody>
</table>
listed in Table 4.1 along with a brief description. Briefly, samples were collected into sterile plastic sampling bottles and transported to the laboratory for processing. The membrane filtration method was used to enumerate total marine bacteria on marine agar (MA) and total vibrios on TCBS and CV agar. Plates were incubated at 35°C and enumerated after 24 hours. Turbidity and salinity measurements of water samples were also taken. Select colonies from CV plates were isolated and were presumptively identified using two biochemical tests: sucrose fermentation and growth at various salt concentrations. Presumptive colonies were confirmed using PCR.

4.4. RESULTS AND DISCUSSION

4.4.1. Total Marine Bacteria and Vibrio Concentrations in the Four Water Quality Categories on the Islands of Oahu and Hawaii

As stated previously, USEPA has not approved methods to enumerate the concentrations of total marine bacteria and total vibrios in marine and estuary environments. This is because USEPA has not determined that a water quality standard for these bacteria in coastal waters is needed. Table 4.2 summarizes the geometric mean concentrations of total marine bacteria, total vibrio bacteria on both TCBS and CV agars and turbidity and salinity measurements of samples from the four water quality categories. In general, turbidity values were highest in categories known to be impacted by land runoff. A turbidity level of >5 NTU was used to determine when excessive sediments were entering coastal waters.

4.4.1.1. Primary Coastal Beaches on Oahu

Primary coastal beaches had a turbidity level of 2.2 NTU and salinity of 35 ppt, indicating that these sites are not significantly impacted by land runoff. Concentrations
of marine bacteria were $3.4 \times 10^4$ CFU/100ml and concentration of vibrio bacteria were similar on both TCBS and CV agar ($9.9 \times 10^3$ and $9.0 \times 10^3$ CFU/100ml, respectively). Marine agar is a nonselective agar, thus most marine bacteria are capable of growth on this media. Thus, of the three types of media used in this study, marine agar is expected to have the highest counts of bacteria. Vibrio counts were not significantly different on both TCBS and CV agars indicating that CV agar was capable of detecting these bacteria at levels similar to that of traditional TCBS media.

**4.4.1.2. Secondary Coastal Beaches on Oahu**

In contrast to primary coastal beaches, secondary coastal beaches had a turbidity of level of 4.6 NTU, which was more than twice as high as primary coastal beaches.

<table>
<thead>
<tr>
<th>Designated Categories Of Coastal Waters</th>
<th>n</th>
<th>Total Marine Bacteria (CFU/100ml)</th>
<th>Total Vibrio Bacteria (CFU/100ml)</th>
<th>Turbidity (NTU)</th>
<th>Salinity (ppt)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>TCBS</td>
<td>CV</td>
<td></td>
<td></td>
</tr>
<tr>
<td>I. Primary Coastal Beaches on Oahu</td>
<td>20</td>
<td>$3.4 \times 10^4$</td>
<td>$9.9 \times 10^3$</td>
<td>2.2</td>
<td>35</td>
</tr>
<tr>
<td>II. Secondary Coastal Beaches on Oahu</td>
<td>15</td>
<td>$4.2 \times 10^4$</td>
<td>$2.1 \times 10^4$</td>
<td>4.6</td>
<td>21</td>
</tr>
<tr>
<td>III. Coastal Harbors, Canals and Ponds on Oahu</td>
<td>\</td>
<td>\</td>
<td>\</td>
<td>\</td>
<td>\</td>
</tr>
<tr>
<td>A. Harbors</td>
<td>11</td>
<td>$3.6 \times 10^4$</td>
<td>$1.2 \times 10^4$</td>
<td>6.8</td>
<td>34</td>
</tr>
<tr>
<td>B. Canals</td>
<td>7</td>
<td>$1.8 \times 10^5$</td>
<td>$2.3 \times 10^4$</td>
<td>4.2</td>
<td>12</td>
</tr>
<tr>
<td>C. Ponds</td>
<td>5</td>
<td>$8.1 \times 10^4$</td>
<td>$2.0 \times 10^4$</td>
<td>6.4</td>
<td>21</td>
</tr>
<tr>
<td>IV. Coastal Swimming Sites on Hawaii</td>
<td>\</td>
<td>\</td>
<td>\</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A. Kona Area</td>
<td>9</td>
<td>$9.8 \times 10^5$</td>
<td>$3.3 \times 10^4$</td>
<td>7.8</td>
<td>11</td>
</tr>
<tr>
<td>B. Hilo Area</td>
<td>8</td>
<td>$8.8 \times 10^3$</td>
<td>$4.4 \times 10^3$</td>
<td>3.0</td>
<td>21</td>
</tr>
<tr>
<td>C. High Temp., Low Salinity Ponds on Island of Hawaii</td>
<td>4</td>
<td>$7.4 \times 10^3$</td>
<td>$2.1 \times 10^2$</td>
<td>2.1</td>
<td>9</td>
</tr>
</tbody>
</table>

Table 4.2. Geometric mean values of total marine bacteria, total vibrio bacteria on TCBS and CV agar and physical parameters of the four water quality categories.
These sites had a geometric mean salinity of 21 ppt, which along with the high turbidity value, is indicative that these sites are being impacted by land runoff. Concentrations of marine bacteria were $4.2 \times 10^4$ CFU/100ml and counts of vibrios bacteria were similar on both TCBS and CV agar ($2.1 \times 10^4$ and $1.9 \times 10^4$ CFU/100ml, respectively).

Concentrations of total marine bacteria at these sites were not significantly different from concentrations at primary coastal beaches, indicating that salinity did not have an effect on overall concentrations of marine bacteria. Vibrio concentrations at these impacted sites, detected on both TCBS and CV agar, were significantly different from concentrations of these bacteria at primary coastal beaches. These results indicate that changes in salinity may have a significant impact on concentrations of vibrio bacteria.

### 4.4.1.3. Coastal Harbors, Ponds and Canals on Oahu

Coastal harbor, canal and pond sites on Oahu had turbidity values ranging from 4.2-6.8 NTU with variable salinities ranging from 12-34 ppt. Harbor sites were characterized by high and variable turbidity readings as well as variable salinity values. These results indicate variable pollution by land-based waters with elevated turbidities and variable salinities. Harbors are known to contain inputs in addition to land runoff such as discharges from boats. These discharges may stimulate the growth of organisms in these waters leading to a higher abundance and an increase in turbidity of water. A second explanation may be that boats entering and leaving the harbor can disturb settled particulates and lead to high turbidity values. Canal and pond sites had high turbidity and low salinities indicating that these sites are impacted by land runoff.

Concentrations of marine bacteria at harbor sites were $3.6 \times 10^4$ CFU/100ml and counts of vibrios bacteria were similar on both TCBS and CV agar ($1.2 \times 10^4$
Numbers of total marine bacteria at these sites were not significantly different from concentrations at primary or secondary coastal beaches, once again indicating that salinity may not have an effect on overall concentrations of marine bacteria. Vibrio concentrations at these sites, detected on both TCBS and CV agar, were slightly higher than concentrations in primary swimming beaches but lower than secondary swimming beaches. This suggests that variable salinity and nutrient levels can affect concentrations of certain populations of marine bacteria. Marine bacteria concentrations were higher in canal (1.8 x 10^5 CFU/100ml) and pond (8.1 x 10^4 CFU/100ml) sites as compared to harbor sites. This was also true for concentrations of vibrios detected on both TCBS and CV agars. Vibrio concentrations in canal sites were 2.3 x 10^4 and 2.7 x 10^4 CFU/100ml on TCBS and CV, respectively. Concentrations in pond sites were 2.0 x 10^4 and 3.1 x 10^4 CFU/100 ml on TCBS and CV, respectively. The relatively high counts of marine bacteria in canal sites may be due to the introduction of nutrients via land runoff. There may be certain bacteria in these sites that are capable of utilizing these nutrients and multiplying.

### 4.4.1.4. Coastal ponds on the Island of Hawai‘i

Coastal swimming sites on the Island of Hawai‘i consisted mainly of low salinity ponds, some of which are thermally heated by underground lava. Swimming sites on this island differ from swimming beaches on Oahu due to differences in the hydrogeology of the two islands. As a result, Oahu beaches represent typical swimming beaches which are sandy and are not influenced by groundwater discharge. In contrast, swimming ponds on the Island of Hawai‘i are highly influenced by groundwater discharge which flows through the island’s porous lava rock into coastal areas. This leads to coastal waters that
have low salinities. Coastal swimming ponds that were sampled in the Kona and Hilo areas were characterized by salinities of 11 and 21 ppt, respectively, indicating that these coastal ponds are impacted by ground water discharge. Turbidities between the two areas were significantly different. Kona area ponds had a geometric mean turbidity value of 7.8 NTU while Hilo area ponds had a turbidity value of 3.0 NTU. This difference in turbidity between the two sides of the island may have to do with rainfall. The Kona area is typically the drier part of the island while the there is generally more rainfall in the Hilo area. The increased rainfall in the Hilo area may serve to flush out particulates from these ponds leading to less turbid ponds. Another explanation may be that because most ponds in the Kona area are anchialine in nature, i.e., they are connected to the ocean via underground connections, there may be less circulation with clean ocean water, making these ponds more turbid. In general, marine bacteria counts were significantly higher at Kona ponds (9.8 x 10⁵ CFU/100 ml) as compared to Hilo ponds (8.8 x 10³ CFU/100 ml). This difference was also seen with total vibrio counts. Concentrations of vibrio bacteria were significantly higher on both TCBS and CV agar in Kona ponds (3.3 x 10⁴ CFU/100 ml and 1.0 x 10⁵ CFU/100 ml, respectively) as compared to Hilo ponds on TCBS and CV agars (4.4 x 10³ and 6.3 x 10³ CFU/100 ml, respectively). Concentrations of marine bacteria and total vibrios in Hilo ponds were also lower than those in primary swimming beaches on Oahu. This may be due to frequent flushing of these ponds by groundwater discharge.

Four low salinity, high temperature ponds were sampled on the Island of Hawai‘i. All four ponds were located in the Hilo area. These ponds are thermally heated by underground lava and are popular among swimmers. Water temperatures in these
thermal ponds ranged from 31-35°C which is similar to human body temperature. Thus, pathogenic *Vibrio* species present in these warm coastal ponds may be better adapted to causing infections in humans. These sites had low salinity (9 ppt), indicating they are heavily influenced by ground water discharge. Similar to other ponds on the Hilo side of the Island of Hawai‘i, these ponds had a low turbidity of 2.1 NTU. Once again this may be explained by the higher rainfall this part of the island receives. Concentrations of marine bacteria ($7.4 \times 10^3$ CFU/100 ml) in these thermal ponds were not significantly different from concentrations found in non-thermal ponds in Hilo. This indicates that most marine bacteria can tolerate high water temperatures above 30°C. In contrast, there was a significant difference in total vibrio counts on both TCBS and CV agars in these thermal ponds as compared to non-thermal ponds in Hilo. Vibrio concentrations on TCBS and CV were $2.1 \times 10^2$ and $8.5 \times 10^2$ CFU/100 ml, respectively. The lower concentrations of these bacteria in higher temperature thermal ponds as compared to non-thermal Hilo ponds are indicative that only a small portion of these bacteria can tolerate water temperatures above 30°C. Those bacterial cells that can tolerate these high temperatures may have a selective advantage when it comes to infecting humans because they can tolerate temperatures comparable to human body temperature.

Based on data presented in Table 4.2, is it apparent that when salinities of samples were high (>21 ppt), counts of total vibrio on TCBS were higher than counts on CV agar. However, when salinities were <21 ppt, counts of total vibrio on CV were higher than on TCBS. This may be because CV media may become less selective as water salinity decreases; however, more samples may have to be analyzed to confirm this.
4.4.2. Prevalence of Human Pathogenic *Vibrio* Species in the Four Water Quality Categories on the Islands of Oahu and Hawaii

The isolates of vibrio colonies on CV agar were speciated and this data was used to determine the prevalence of human pathogenic *Vibrio* spp. in the four categories of coastal water. Table 4.3 summarizes the prevalence of pathogenic *Vibrio* spp. (*V. cholerae, V. vulnificus, V. parahaemolyticus* and *V. alginolyticus*) in samples from the four water quality categories.

4.4.2.1. Primary Coastal Beaches on Oahu

At primary swimming beaches, *V. alginolyticus* was the only species detected, and it was prevalent in all 20 sampling beaches. This is consistent with known data. It is known that this species can tolerate high salinities and is distributed worldwide. *V. cholerae, V. vulnificus* and *V. parahaemolyticus* were not prevalent at any of the sampled primary swimming beach sites. *V. cholerae* is known to prefer low salinities and are recovered in waters with salinities below 10 ppt. It should be noted that in studies conducted in other parts of the world *V. cholerae* has been successfully recovered. However many of these studies use a pre-enrichment step in alkaline peptone water (APW) prior to culture or molecular detection (Koch et al., 1993; Barbieri et al., 1999; Blackstone et al., 2007). Pre-enrichment using a selective broth like APW select for the growth of *V. cholerae* cells which may otherwise go undetected due to the fact that they are present in low numbers in the environment. *V. vulnificus* is generally recovered in salinities ranging from 8 to 23 ppt while *V. parahaemolyticus* is tolerant of salinities greater than 15 ppt. Because primary swimming beaches had a salinity above the
tolerance ranges of *V. cholerae*, *V. vulnificus* and *V. parahaemolyticus*, these three species were not prevalent at these sites.

### 4.4.2.2. Secondary Coastal Beaches on Oahu

At secondary swimming beaches, *V. alginolyticus* was the most prevalent species and was recovered from all 15 beach sites. Though this species is generally associated with high salinity waters, nutrients brought in by land run-off can overcome salinity barriers and stimulate *Vibrio* species growth. *V. vulnificus* was recovered in 40% of (or 6 out of 15) sites while *V. parahaemolyticus* was recovered in 20% of (or 3 out of 15) sites.

Table 4.3. The prevalence of *V. cholerae* (*Vc*), *V. vulnificus* (*Vv*), *V. parahaemolyticus* (*Vp*) and *V. alginolyticus* (*Va*) in four water quality categories of Hawaii.

<table>
<thead>
<tr>
<th>Designated Categories Of Coastal Waters</th>
<th>n</th>
<th>Prevalence of <em>Vc</em> % (Positive Sites)</th>
<th>Prevalence of <em>Vv</em> % (Positive Sites)</th>
<th>Prevalence of <em>Vp</em> % (Positive Sites)</th>
<th>Prevalence of <em>Va</em> % (Positive Sites)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I. Primary Coastal Beaches on Oahu</td>
<td>20</td>
<td>0(0)</td>
<td>0</td>
<td>0</td>
<td>100(20)</td>
</tr>
<tr>
<td>II. Secondary Coastal Beaches on Oahu</td>
<td>15</td>
<td>0(0)</td>
<td>40(6)</td>
<td>20(3)</td>
<td>100(15)</td>
</tr>
<tr>
<td>III. Coastal Harbors, Canals and Ponds On Oahu</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A. Harbors</td>
<td>11</td>
<td>0(0)</td>
<td>0(0)</td>
<td>45(5)</td>
<td>100(11)</td>
</tr>
<tr>
<td>B. Canals</td>
<td>7</td>
<td>0(0)</td>
<td>57(4)</td>
<td>71(5)</td>
<td>100(7)</td>
</tr>
<tr>
<td>C. Ponds</td>
<td>5</td>
<td>0(0)</td>
<td>20(1)</td>
<td>40(2)</td>
<td>100(5)</td>
</tr>
<tr>
<td>IV. Coastal Swimming Sites on Hawaii</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A. Kona Area</td>
<td>9</td>
<td>0(0)</td>
<td>22(2)</td>
<td>22(2)</td>
<td>89(8)</td>
</tr>
<tr>
<td>B. Hilo Area</td>
<td>8</td>
<td>0(0)</td>
<td>38(3)</td>
<td>50(4)</td>
<td>100(8)</td>
</tr>
<tr>
<td>C. High Temp., Low Salinity Ponds on Island of Hawaii</td>
<td>4</td>
<td>0(0)</td>
<td>100(4)</td>
<td>75(3)</td>
<td>100(4)</td>
</tr>
</tbody>
</table>
In contrast to primary swimming beaches, secondary swimming beaches were characterized by lower salinity. Thus, it is not surprising that both *V. vulnificus* and *V. parahaemolyticus* were prevalent at these swimming beaches. In addition, within this category, *V. vulnificus* was prevalent at sites that had salinities ranging from 6 to 25 ppt while *V. parahaemolyticus* was prevalent at sites that had salinities greater than 21 ppt. This is in agreement with what is known about these two species. *V. vulnificus* is generally recovered in salinities ranging from 8 to 23 ppt while *V. parahaemolyticus* is tolerant of salinities greater than 15 ppt. *V. cholerae* was not recovered at any of the secondary swimming beaches which had salinities that were greater than those that can be tolerated by this species.

### 4.4.2.3. Coastal Harbors, Ponds and Canals on Oahu

At harbor, canal and pond sites, *V. alginolyticus* was the most prevalent species and was recovered from all 23 sampling sites. This species is generally associated with high salinity waters, however nutrients brought in by land run-off can overcome salinity barriers and stimulate *Vibrio* species growth. At harbor sites, *V. vulnificus* was not recovered at any of the sites while *V. parahaemolyticus* was prevalent in 45% of (or 5 out of 11) sites. These sites were characterized by high salinity which may be why *V. parahaemolyticus* and not *V. vulnificus* was prevalent at these sites. Both species were recovered in lower salinity canal and pond sites. *V. vulnificus* was prevalent in 57% of (or 4 out of 7) canal sites and 20% of (or 1 out of 5) pond sites. *V. parahaemolyticus* was prevalent in 71% of (5 out of 7) canal sites and 40% of (or 2 out of 5) pond sites. This is
in agreement with what is known about the salinity tolerance of these two species. *V. cholerae* was not recovered at harbor, canal or pond sites which had salinities that were greater than those that can be tolerated by this species.

### 4.4.2.4. Coastal ponds on the Island of Hawaiʻi

At both Kona and Hilo ponds, *V. alginolyticus* was once again the most prevalent *Vibrio* species recovered. It was recovered in 89% of (8 out of 9) Kona ponds and 100% of Hilo ponds. Both *V. vulnificus* and *V. parahaemolyticus* were prevalent in both Kona and Hilo ponds. *V. vulnificus* was prevalent in 22% of (or 2 out of 9) Kona ponds and 38% of (or 3 out of 8) Hilo ponds. *V. parahaemolyticus* was prevalent in 22% of (or 2 out of 9) Kona ponds and 50% of (or 4 out of 8) Hilo ponds. These ponds have low salinity due to impact by ground water run-off. Thus, the prevalence of *V. vulnificus* and *V. parahaemolyticus* is in agreement with what is known about the salinity tolerance of these two species. *V. cholerae* was not recovered in Kona or Hilo ponds which had salinities greater than those that can be tolerated by this species.

*V. alginolyticus* was the most prevalent *Vibrio* species recovered from high temperature, low salinity ponds on the Island of Hawaiʻi. This species was prevalent in all four thermal ponds which is indicative that these pathogens can tolerate high water temperatures as well as low salinity. In addition, *V. vulnificus* was recovered from all four thermal ponds, and *V. parahaemolyticus* was recovered from 75% of (or 3 out of 4) of these ponds. The prevalence of *V. vulnificus* and *V. parahaemolyticus* is in agreement with what is known about the salinity tolerance of these two species. In addition, death resulting from *V. vulnificus* infection has occurred at one of these sites. This is indicative that species of pathogenic *Vibrio* species present in these thermal ponds may be more
virulent to humans because they are adapted to growing at temperatures comparable to human body temperature.

4.5. SUMMARY AND CONCLUSIONS

The current knowledge on the ecology and survival of *Vibrio* species comes from studies conducted in temperate areas. These studies have shown that water temperature and salinity are two environmental factors that influence the abundance and growth of this genus. Thus far, similar studies have been lacking in tropical regions where water temperatures are almost constant year round. This phase of the study had two objectives. The first objective of this phase of study was to determine the prevalence of four human pathogenic *Vibrio* species in the four categories of coastal waters based on expected water salinity and expected impact by contamination by land based run-off. The second objective of this phase was to determine whether environmental conditions that had been previously reported to control for the growth of the four human pathogenic *Vibrio* spp. can be used to explain the results when water samples from the four categories of coastal water analyzed. Based on data gathered from studies conducted in temperate areas, we hypothesized that, because pathogenic *Vibrio* species are known to prefer lower salinity for growth, we would expect them to be present in low salinity waters that are impacted by land runoff. We found this to be true in the case of *V. vulnificus* and *V. parahaemolyticus*. These two species were prevalent in low salinity sites that were impacted by land run-off but not detectable in high salinity, non-impacted swimming sites. *V. alginolyticus* was prevalent in all sites regardless of salinity. Though this species is generally associated with high salinity waters, nutrients brought in by land run-off may overcome salinity barriers and stimulate the growth of this *Vibrio* species.
Furthermore, *V. vulnificus* and *V. parahaemolyticus* were prevalent at low salinity swimming ponds on the Island of Hawaii, indicating once again that salinity has a strong influence on the prevalence of these two species. *V. cholerae* was not recovered in either impacted or non-impacted sites. Studies have successfully recovered this species in coastal waters, however, detection typically follows an enrichment step prior to culture. Enrichment of samples was not done in this study, thus this species may have been below the detection limit of our assay. In addition, *V. cholerae* has been found in close association with chitinous organisms such as zooplankton. Studies have shown that this association is used by this species during times of environmental stress (i.e. low water temperature), therefore it may be possible that because these sites had salinities above those which are tolerable by *V. cholerae* they may be more likely to be attached to zooplankton than in the water column. Thus, based on our data we can conclude that our hypothesis was valid and that *V. vulnificus* and *V. parahaemolyticus* are more prevalent in low salinity coastal waters that are impacted by land run-off.

Data from this study also indicate that similar to land runoff impacted beach sites, *V. alginolyticus*, *V. vulnificus* (with the exception of harbor sites) and *V. parahaemolyticus* were prevalent at harbor, canal and pond sites. *V. vulnificus* was not recovered from any of the 11 harbor sites which may be explained by the high salinity (34 ppt) at these sites. Once again, *V. cholerae* was not recovered from any of the sampling sites. We hypothesized that because these sites are not flushed out by clean ocean waters, they contain elevated nutrients and lower salinity; conditions that favor the growth of *Vibrio* species. Thus, based on our data we can conclude that our hypothesis
was valid and that \textit{V. vulnificus} and \textit{V. parahaemolyticus} were prevalent in these low salinity, stagnant coastal waters.

Based on our data we also know that low salinity and warm water temperatures (>30°C) present on the Island of Hawaii are selective for the growth of the four human pathogenic \textit{Vibrio} species. We hypothesized that because these conditions are known to favor the growth of pathogenic \textit{Vibrio} species, we would expect them to be present in thermally heated ponds present on the Island of Hawaii. We found that though overall counts of bacteria were lower in these ponds as compared to non-thermally heated ponds, \textit{V. alginolyticus, V. vulnificus} and \textit{V. parahaemolyticus} were prevalent in these thermal ponds. Thus we can conclude that our hypothesis was valid and that high water temperature and low salinity can be selective for these three \textit{Vibrio} pathogens. Furthermore, there has been past evidence of infection and death due to \textit{V. vulnificus} associated with the use of these ponds. Thus, isolates recovered from these areas may potentially be more virulent as they have been adapted to survival at temperatures similar to that of human body temperature.
CHAPTER 5
THE PREVALENCE OF PATHOGENIC VIBRIO SPECIES IN BIOFILM SEDIMENTS AND HUMAN SEWAGE

5.1. INTRODUCTION

Little is known about the distribution, abundance, survival and ecological roles of Vibrio species in marine sediments (Urakawa and Rivera, 2006). Studies have shown that V. parahaemolyticus can be recovered from sediment samples throughout the year (Kaneko and Colwell, 1973), indicating that this may be a potential reservoir of Vibrio species. Furthermore, this study indicated that though levels of Vibrio species were relatively constant in sediment bottom water temperature did influence their concentrations. V. parahaemolyticus counts were found to be $10^4$ cells per gram of sediment when water temperatures were $>20^\circ$C but dropped down to $10^2$ cells per gram of sediment when water temperatures were cooler $(6^\circ$C). Thus, in temperate areas, the increase and decrease of Vibrio species in sediments follows a similar seasonal trend as seen in the water column. One hypothesis for the decreased detection of these species in the water column during the cooler months, followed by an increase in numbers during warmer months is, these bacteria aggregate in sediments, and during mixing events become resuspended into the water column. Furthermore, it is know that bacteria can more easily obtain nutrients when attached to particles as opposed to freely floating in the water column. Thus, we would expect to find higher concentrations of bacteria, and Vibrio species, in sediments as opposed to the water column and these sediments can serve as a continuous reservoir for these pathogens.

Everyday millions of gallons of treated sewage are released into our oceans. Accidental release of untreated sewage has also known to occur. These processes may be
introducing pathogenic bacteria, including pathogenic *Vibrio* species, into our coastal waters. These pathogens can then potentially cause infections to recreational users of these waters. Sewer surveillances conducted in Guam and Louisiana detected the presence of toxigenic strains of *V. cholerae* 01 in areas where no cases of cholera had been detected by surveillance of people seeking medical attention due to diarrheal disease (Barrett et al., 1980). This may be due to high numbers of asymptomatic individuals within populations. It has been estimated that the ratio of asymptomatic to symptomatic infections can be as high as 250:1 (King et al., 2008). In addition, a study found that *Vibrio* species could be detected in chlorine disinfected effluent at $10^3$ CFU/ml (Igbinosa et al., 2009). This same study demonstrated that pathogenic *Vibrio* species, including *V. cholerae*, *V. vulnificus* and *V. parahaemolyticus*, were prevalent in treated effluent. Thus, accidental sewage spills and even normal discharge of treated effluent may serve as a source of release of pathogenic *Vibrio* species into the environment.

5.2. STUDY OBJECTIVES

**Objective 1:** To determine the prevalence of the four human pathogenic *Vibrio* spp. in sediment biofilm of coastal waters as a source of these pathogens in the water column.

**Hypothesis:** because *Vibrio* species can more consistently obtain nutrients in sediment biofilm than in the water column, sediments of coastal waters can be expected to be sources of pathogenic *Vibrio* spp.

**Objective 2:** To determine whether raw sewage, primary sewage and final disinfected sewage can serve as possible sources of the four human pathogens
Vibrio spp. when discharged into coastal waters.

**Hypothesis:** pathogenic Vibrio species have been recovered in raw and treated sewage, therefore humans infected by these pathogens are likely to shed these bacteria into sewage which can serve as a possible source of pathogens into coastal waters.

### 5.3. EXPERIMENTAL DESIGN AND METHODS

Approximately 500 g of sediment was collected from primary and secondary swimming beaches on Oahu. Sediments were collected at knee depth using a strainer overlaid with a nylon mesh. This allowed excess water to drain and the sediment to retain in the strainer. Collected sediment was placed into sterile Whirl-Pak bags and transported to the lab for analysis. Three samples were taken from each beach site and pooled for analysis. Bacteria from sediments were eluted using a modified procedure used by the USGS. Briefly, 100 g of well mixed sediment was placed into a sterile, 500 ml plastic bottle. 200 ml was of peptone buffer (PB) was added and the bottle was hand shaken for 5 minutes. The samples were allowed to settle for 30 seconds and the eluate was collected. This elution process was done twice for each sample. The eluates were processed in a similar manner as water samples. Membrane filtration was used to enumerate total marine bacteria on marine agar (MA) and total vibrios on TCBS and CV agar. Plates were incubated at 35°C and enumerated after 24 hours. Select colonies from CV plates were isolated and were presumptively identified using two biochemical tests: sucrose fermentation and growth at various salt concentrations. Presumptive colonies were confirmed using PCR.
Sewage samples were obtained from three sewage treatment plants on Oahu: Sand Island Wastwater Treatment Plant (WTP), Hawaii Kai WTP and Wahiawa WTP. Typically each treatment plant differs in the methods used to treat their sewage. Table 5.1 lists the three treatment plants and the methods used for primary and final treatment of sewage.

Sewage samples were collected in sterile, 500 ml plastic bottles from three sewage treatment plants. Each treatment plant was visited three times and raw, primary treated and final treated effluent was collected. Samples were collected using a sterile sampling bucket and transported to the laboratory for analysis. Membrane filtration was used to enumerate total marine bacteria on marine agar (MA) and total vibrios on TCBS and CV agar. Plates were incubated at 35°C and enumerated after 24 hours. In the case of raw and primary treated sewage samples, samples had to be diluted 10,000-fold until a countable plate on CV was obtained. As a result, colonies for confirmation came from undilute CV plates. However, because these undilute plates did not have distinguishable colonies, sterile toothpicks were used to scrape off growth from the membranes which were streaked for isolation on four fresh CV plates. Twenty colonies from each plate was picked and confirmed biochemically and with PCR.

Table 5.1. Primary and final treatment methods used to treat sewage influent at Sand Island, Hawaii Kai and Wahiawa Wastewater Treatment Plants.

<table>
<thead>
<tr>
<th>Treatment Plant</th>
<th>Primary Treatment</th>
<th>Final Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sand Island WTP</td>
<td>Settling tank</td>
<td>UV disinfection</td>
</tr>
<tr>
<td>Hawaii Kai WTP</td>
<td>Settling tank</td>
<td>Chlorination</td>
</tr>
<tr>
<td>Wahiawa WTP</td>
<td>Sand filtration</td>
<td>UV disinfection</td>
</tr>
</tbody>
</table>
5.4. RESULTS AND DISCUSSION

5.4.1. Sediment Biofilm

5.4.1.1. Visual Observation of Sand Sediment

Sediment samples from primary and secondary coastal beaches were visually different. Those from primary beaches were generally light tan to brown in appearance while sediments from secondary beaches were darker in color and consisted of finer particles. Because these secondary sites are impacted by runoff, these particles are most likely deposits that were brought from nearby streams and other land contamination. In addition, during the elution process the finer particles in secondary beach sediment did not settle as easily and generally remained in the liquid phase.

5.4.1.2. Microbial Concentrations

Concentrations of total marine bacteria and total vibrio bacteria in primary and secondary coastal beaches are listed in Table 5.2. Secondary coastal beach sediments contained significantly higher counts of marine bacteria \((1.9 \times 10^6 \text{ CFU/100 g})\) than primary beach sediments \((3.3 \times 10^5 \text{ CFU/100 g})\). In addition, sediment concentrations of these bacteria were 10 to 100 fold higher than concentrations in the water column. Total vibrio concentrations were also much higher in secondary beach sediments \((2.6 \times 10^5 \text{ CFU/100g on TCBS and } 3.4 \times 10^5 \text{ CFU/100g on CV})\) as compared to primary beach sediments \((7.0 \times 10^4 \text{ CFU/100g on TCBS and } 1.5 \times 10^5 \text{ CFU/100 g on CV})\) indicating that water salinity and land-run off can impact vibrio concentrations even in sediments. As with concentrations of marine bacteria, total vibrio concentrations were 10 to 100 fold higher in sediments as compared with what was observed in the water column. However,
Table 5.2. Geometric mean values of total marine bacteria and total vibrio bacteria in sediment biofilms from primary and secondary coastal beaches.

<table>
<thead>
<tr>
<th>Name of Category</th>
<th>n</th>
<th>Total Marine Bacteria (CFU/100g)</th>
<th>Total Vibrio Bacteria (CFU/100g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I. Primary Coastal Beaches on Oahu</td>
<td>13</td>
<td>3.3 x 10^5</td>
<td>7.0 x 10^4</td>
</tr>
<tr>
<td>II. Secondary Coastal Beaches on Oahu</td>
<td>10</td>
<td>1.9 x 10^6</td>
<td>2.6 x 10^5</td>
</tr>
</tbody>
</table>

Unlike with water samples from primary and secondary beaches, sediments from these beach categories yielded higher vibrio counts on CV than on TCBS. This may indicate that CV agar may be better for enumerating vibrio bacteria in sediment samples.

5.4.1.3. Prevalence of Vibrio Species

The trend of pathogenic Vibrio species prevalence in sediment samples followed a similar trend as what was observed with water samples. Table 5.3 lists the prevalence of these four pathogens in primary and secondary coastal beach sediments on Oahu.

Table 5.3. The prevalence of V. cholerae (Vc), V. vulnificus (Vv), V. parahaemolyticus (Vp) and V. alginolyticus (Va) in primary and secondary coastal beach sediments on Oahu.

<table>
<thead>
<tr>
<th>Name of Sediment Category</th>
<th>n</th>
<th>Prevalence of Vc % (Positive Sites)</th>
<th>Prevalence of Vv % (Positive Sites)</th>
<th>Prevalence of Vp % (Positive Sites)</th>
<th>Prevalence of Va % (Positive Sites)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I. Primary Coastal Beaches on Oahu</td>
<td>13</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>100 (13)</td>
</tr>
<tr>
<td>II. Secondary Coastal Beaches on Oahu</td>
<td>10</td>
<td>0</td>
<td>60(6)</td>
<td>50 (5)</td>
<td>100 (10)</td>
</tr>
</tbody>
</table>

V. alginolyticus was prevalent in 100% of sediment samples from both primary and secondary coastal beaches. V. vulnificus and V. parahaemolyticus was not recovered in primary beach sediment but was prevalent in secondary beach sediment.
was present in 60% of (or 6 out of 10) secondary beach sediment sites while \textit{V. parahaemolyticus} was present in 50% of (or 5 out of 10) secondary beach sediment sites. \textit{V. cholerae} was not detected in any of the sediment samples once again implying that this species may occupy a habitat in the marine environment that is different from other pathogenic \textit{Vibrio} species. The high prevalence of \textit{V. vulnificus} and \textit{V. parahaemolyticus} in secondary beach sediments is indicative that once these pathogens enter these environments they can persist in sediments. The low salinity and high nutrient load brought in by land run-off is conducive to the persistence of these pathogens at secondary beach sites. In addition, these pathogens can become reintroduced from the sediments into the water column through wave action or by swimmers and waders who disturb the sediment. Thus sediment habitats can serve as sources of pathogenic \textit{Vibrio} species.

5.4.2. The Recovery of Pathogenic \textit{Vibrio} Species From Raw, Primary Treated and Final Treated Effluent

Three sewage treatment processes from three different sewage treatment plants on Oahu were assayed for the four human pathogenic \textit{Vibrio} species. The results of the presence of these pathogens are listed in Table 5.4. The Sand Island and Hawaii Kai WTPs were characterized by higher salinities (5-7 ppt and 4 ppt, respectively) as compared to Wahiawa WTP (0 ppt). These two plants are located along the coast line, thus, subsurface salt water intrusion into the sewer lines may contribute to the higher salinities of the sewage seen at these plants. In contrast, the Wahiawa WTP is located inland and is not influenced by salt water intrusion.

\textit{V. alginolyticus} was not detected in any of the sewage treatments at any of the treatment plants. \textit{V. vulnificus} and \textit{V. parahaemolyticus} were sporadically recovered from raw and primary treatments from all three treatment plants. This may indicate that sporadic shedding of these two \textit{Vibrio} pathogens are occurring within the population. \textit{V. cholerae} on the other hand was consistently recovered in raw and primary treated sewage from all three sewage treatment plants. This data is consistent with reported data that
Vibrio species can be recovered from human sewage and that *V. cholerae* can be present in sewage even when no cases are apparent in the community. This can be attributed to asymptomatic infections by this species. Thus, accidental sewage spills can contribute to the release of these pathogens into the environment, which can constitute a health risk to the public. None of the *Vibrio* pathogens were recovered in final treated sewage which is ultimately what becomes discharged into the marine environment. However, it is known that a certain portion of pathogens can survive the disinfection process. Therefore, combined with the large volumes of treated sewage being discharged, it is likely that a portion of *Vibrio* pathogens may be released into the environment on a daily basis.

Table 5.4. The presence of *V. cholerae* (*Vc*), *V. vulnificus* (*Vv*), *V. parahaemolyticus* (*Vp*) and *V. alginolyticus* (*Va*) in three wastewater treatment plants on Oahu (“+” indicates *Vibrio* species was detected while “-“ indicates *Vibrio* species was not detected).

<table>
<thead>
<tr>
<th>Treatment Plant</th>
<th>Salinity (pp)</th>
<th><em>Vc</em></th>
<th><em>Vv</em></th>
<th><em>Vp</em></th>
<th><em>Va</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Sand Island</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Raw</td>
<td>7</td>
<td>+/-+</td>
<td>-/-</td>
<td>-/-</td>
<td>-/-</td>
</tr>
<tr>
<td>Primary Treated</td>
<td>5</td>
<td>-/++</td>
<td>-/++</td>
<td>-/+-</td>
<td>-/-</td>
</tr>
<tr>
<td>Final Treated</td>
<td>5</td>
<td>-/-</td>
<td>-/-</td>
<td>-/-</td>
<td>-/-</td>
</tr>
<tr>
<td>Hawaii Kai</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Raw</td>
<td>4</td>
<td>+/-+</td>
<td>+/-</td>
<td>+/-</td>
<td>-/-</td>
</tr>
<tr>
<td>Primary Treated</td>
<td>4</td>
<td>+/-+</td>
<td>+/-</td>
<td>+/-</td>
<td>-/-</td>
</tr>
<tr>
<td>Final Treated</td>
<td>4</td>
<td>-/-</td>
<td>-/-</td>
<td>-/-</td>
<td>-/-</td>
</tr>
<tr>
<td>Wahiawa</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Raw</td>
<td>0</td>
<td>+/-+</td>
<td>-/-</td>
<td>+/-</td>
<td>-/-</td>
</tr>
<tr>
<td>Primary Treated</td>
<td>0</td>
<td>+/-+</td>
<td>-/-</td>
<td>+/-</td>
<td>-/-</td>
</tr>
<tr>
<td>Final Treated</td>
<td>0</td>
<td>-/-</td>
<td>-/-</td>
<td>-/-</td>
<td>-/-</td>
</tr>
</tbody>
</table>
5.5. SUMMARY AND CONCLUSIONS

The overall goal of this phase of the study was to determine sources of pathogenic *Vibrio* species into coastal waters. The first objective was to determine the prevalence of *V. cholerae*, *V. vulnificus*, *V. parahaemolyticus* and *V. alginolyticus* in sediment biofilm of coastal waters and to determine if sediments could serve as a source of these pathogens into the water column. We hypothesized that because *Vibrio* species can more consistently obtain nutrients in sediment biofilm than in the water column, sediments of coastal waters can be expected to be sources of pathogenic *Vibrio* species. Data from this study showed that the prevalence of pathogenic *Vibrio* species in sediments followed a similar trend to what was seen with coastal beach samples. *V. alginolyticus* was prevalent in both primary and secondary beach sediment while *V. vulnificus* and *V. parahaemolyticus* was only prevalent in secondary beach sediment. These sites have lower salinity and are influence by land run-off and these two species were also prevalent in the water columns of these types of sites. Based on data from this study it is apparent that sediments from secondary coastal waters can serve as a source of pathogenic *Vibrio* species into the water column. Thus, our hypothesis is valid.

The second objective was to determine whether raw sewage, primary treated sewage and final disinfected sewage could serve as possible sources of the four human pathogenic *Vibrio* species when discharged into coastal waters. We hypothesized that because pathogenic *Vibrio* species have been previously recovered in raw and treated sewage, infected humans are likely to shed these bacteria into sewage which can then serve as a possible source of contamination to coastal waters. Data from this study showed that *V. vulnificus* and *V. parahaemolyticus* were sporadically present in raw and
primary treated sewage from three different wastewater treatment plants. *V. cholerae* on the other hand was consistently recovered in raw and primary treated sewage from all three treatment plants. Based on this data it is apparent that sporadic shedding of *V. vulnificus* and *V. parahaemolyticus* is occurring within the population, while *V. cholerae* is being consistently shed by asymptomatic carriers. However, none of the pathogenic *Vibrio* species were present in the samples analyzed from disinfected effluent. Thus, only accidental release of untreated sewage can introduce these pathogens into coastal waters which may lead to public health consequences to users of these waters. Based on this data we can say that our hypothesis is valid. Furthermore, two of the largest sewage treatment plants on Oahu (Sand Island and Honouliuli) have previously discharged primary treated and non-disinfected sewage into the ocean. This may have potentially lead to the release of *V. cholerae* strains into the environment capable of infecting human hosts.
CHAPTER 6
THE PRESENCE OF VIRULENCE DETERMINANTS IN VIBRIO SPECIES ISOLATED FROM COASTAL WATERS, SEDIMENTS AND SEWAGE

6.1. INTRODUCTION

The possession of virulence determinants by a microorganism has long been thought to give these organisms a competitive advantage in terms of survival and the ability to cause infection. Often these determinants are used to classify organisms as virulent or non-virulent. However, with some microorganisms, particularly, those which are naturally found in the environment, the role of virulence determinants is not as clear. Often, the main functional role of these organisms is not to cause disease in humans but rather to fulfill a functional niche in the environment (i.e. breakdown of complex molecules). Such species, like Vibrios, however are known to cause opportunistic infections in a variety of aquatic animals as well as humans.

Several virulence determinants have been recognized in Vibrio species, and standard PCR technologies are often used in the detection of these virulence factors. In the case of V. cholerae, the gene encoding cholera toxin production is often targeted. This toxin is produced by toxigenic strains of V. cholerae and is responsible for the main clinical symptoms of cholera disease (i.e. diarrhea and fluid loss). The presence of gene sequences similar to pandemic serotypes of V. cholerae (O1, O139), are also often the target of these assays. Strains of V. cholerae that do not produce cholera toxin (non-toxigenic strains) are still capable of causing gastrointestinal symptoms in humans, though these symptoms are generally milder than cholera.
In the case of *V. vulnificus*, early studies showed that a hemolysin, termed cytolysin-hemolysin was responsible for its pathogenicity. Evidence now however shows that this hemolysin may not play a significant role in *V. vulnificus* pathogenicity (Harwood et al., 2004). Instead, genetic markers have been developed that are capable of distinguishing between environmental and clinical strains of this species. This typing is based on differences in 16S rRNA sequences between strains isolated from environmental (type A) and clinical (type B) settings. A study conducted by Kim and Jeong (2001) analyzed 40 isolates of *V. vulnificus* from oysters, seawater and mud and found that 35% were of the A type and 65% were of the B type. It was concluded from this study that the original characterization of *V. vulnificus* isolates into biotypes, based on biochemical properties, did not reflect the genetic heterogeneity of this species. A more recent study conducted in Galveston Bay (Texas) showed that concentrations of type A and type B strains varied with water temperature. As expected based on previous studies, neither types were detected when water temperatures fell below 15°C. However, as water temperatures started to warm between March and May (15-20°C), type A was more predominant while between June and September when water temperatures were 20 to 25°C, type B was predominant (Lin and Schwarz, 2003). Thus, in warm tropical areas like Hawaii where water temperatures range between 24-27°C year round, we would expect type B strains to be the dominant subtype of *V. vulnificus*.

The main target for pathogenic strains of *V. parahaemolyticus* has been the genes involved in the production of two hemolysins: TDH and TRH. TDH has been associated with isolates that exhibit the Kanagawa Phenomenon (ß-hemolysis on Wagatsuma agar) while TRH has been associated with strains that are Kanagawa negative. Detection of
tdh and trh genes using molecular methods have replaced the use of testing strains on Wagatsuma agar because they are more rapid and some V. parahaemolyticus strains have shown weak hemolytic activity on this agar (Nishibuchi et al., 1985). Strains of V. parahaemolyticus isolated from patients with gastrointestinal illness are generally positive for one or both of these hemolysins while genes for these hemolysins are generally not detected in environmental strains.

6.2. STUDY OBJECTIVE

Objective: To characterize isolates obtained from sampling locations for the presence of various virulence determinants.

Hypothesis: virulence determinants should be absent from our strains because it is known that environmental strains of these bacteria generally do not possess genes associated with virulence.

6.3. EXPERIMENTAL DESIGN

All environmental isolates of V. cholerae, V. vulnificus and V. parahaemolyticus obtained from previous phases of this study were further characterized based on the presence/absence of virulence determinants. Because virulence determinants of V. alginolyticus have largely not been characterized, isolates of this species were excluded from this phase of the study. All isolates were grown in 1 ml of TSB + 1.5% NaCl at 35°C for 24 hours and DNA was extracted as previously described. Briefly, cultures were boiled at 100°C on a heat block for 5 minutes and centrifuged for 5 minutes at 10,000 RPM in a microcentrifuge (Lee et al., 2004). The resulting supernatant was used
as template for PCR reactions. Primers targeting species specific virulence determinants were used to detect ctxA, O1 and O139 serotypes among V. cholerae isolates, 16S subtypes among V. vulnificus isolates and hemolysin genes tdh and trh in V. parahaemolyticus isolates. The protocols for PCR amplification described for each set of primers were followed (see Table 3.2 for references) and PCR products were visualized on 1.5% agarose gels.

6.4. RESULTS AND DISCUSSION

The presence of virulence determinants in V. cholerae isolates from the three wastewater treatment plants are described in Table 6.1. Of the 114 total V. cholerae isolates from three wastewater treatment plants, none of the isolates had sequence homology to pandemic serotypes O1 and O139, and only one toxigenic V. cholerae strain was detected. This isolate came from raw sewage obtained from the Hawaii Kai WTP, and was isolated from 25 ml of raw sewage (maximum volume of sewage that could be filtered without clogging the filter). This treatment plant receives more than five million gallons of sewage per day from the Hawaii Kai area. Thus, there may potentially be hundreds of thousands of toxigenic V. cholerae cells present in this sewage, and an accidental sewage spill may release these toxigenic strains into the environment. Furthermore, it is possible that these toxigenic strains of V. cholerae may pass on their virulence genes to nontoxigenic strains in the environment. Toxigenic strains of V. cholerae have been detected in sewer lines in Guam and Louisiana (Barrett et al., 1980).
Table 6.1. The presence of cholera toxin genes (*ctxA*) and genes associated with pandemic strains of cholera (O1 and O139) in *V. cholerae* strains isolated in human sewage from three wastewater treatment plants on Oahu.

<table>
<thead>
<tr>
<th>Isolated Site</th>
<th>No. of Isolates</th>
<th>No. <em>ctxA</em> Positive</th>
<th>No. 01 Positive</th>
<th>No. 0139 Positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sand Island WTP</td>
<td>18</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Hawaii Kai WTP</td>
<td>68</td>
<td>1*</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Wahiawa WTP</td>
<td>28</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>TOTAL</td>
<td>114</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

* detected in raw sewage from Hawaii Kai WTP.

Of a total of 151 confirmed isolates of *V. vulnificus*, 129 (74%) exhibited 16S sequence homology to type B (clinical) strains and the remaining 31 (26%) were not typed as A or B. The reason that 26% of isolates were not typed is unclear. It may be that these isolates contain small base differences in their 16S that prevent them from being identified as either type with the primers that were used. Sequencing these isolates may provide some clue as to why these isolates escape typing. The presence of majority B types of *V. vulnificus* in Hawaii’s coastal waters has also been documented elsewhere (Kim and Jeong, 2001; Lin and Schwarz, 2003). The studies conducted by Lin and Schwarz also indicate that during the cooler months when water temperatures are 15-20°C, *V. vulnificus* type A (environmental subtype) is the predominant type. In contrast, during the warmer months when water temperatures are greater than 20°C *V. vulnificus* type B (clinical subtype) is the predominant type. Because Hawaii’s water temperatures are generally between 24 to 27°C, our warmer temperatures may be selecting for the B subtypes of *V. vulnificus*. 
Table 6.2. Subtyping of *V. vulnificus* strains isolated from Hawaii’s coastal waters as environmental (type A) or clinical (type B) based on 16S profiles.

<table>
<thead>
<tr>
<th>Isolated Site</th>
<th>No. of Isolates</th>
<th>No. Environmental (A) Subtypes (%)</th>
<th>No. Clinical (B) Subtypes (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primary Coastal Beaches on Oahu</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Secondary Coastal Beaches on Oahu</td>
<td>38</td>
<td>0</td>
<td>29(76)</td>
</tr>
<tr>
<td>Coastal Harbors, Canals and Ponds on Oahu</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Harbors</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Canals</td>
<td>10</td>
<td>0</td>
<td>4(40)</td>
</tr>
<tr>
<td>Ponds</td>
<td>3</td>
<td>0</td>
<td>3(100)</td>
</tr>
<tr>
<td>Coastal Swimming Sites on Hawaii</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kona Area</td>
<td>11</td>
<td>0</td>
<td>8(73)</td>
</tr>
<tr>
<td>Hilo Area</td>
<td>7</td>
<td>0</td>
<td>1(14)</td>
</tr>
<tr>
<td>High Temp., Low Salinity Ponds on Island of Hawaii</td>
<td>8</td>
<td>0</td>
<td>1(13)</td>
</tr>
<tr>
<td>Sediments</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Primary Coastal Beaches</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Secondary Coastal Beaches</td>
<td>67</td>
<td>0</td>
<td>59(88)</td>
</tr>
<tr>
<td>Human Sewage</td>
<td>7</td>
<td>0</td>
<td>7(100)</td>
</tr>
<tr>
<td>TOTAL</td>
<td>151</td>
<td>0</td>
<td>112(74)</td>
</tr>
</tbody>
</table>

Of a total of 160 confirmed isolates *V. parahaemolyticus*, one isolate was positive for the gene involved in thermostable direct hemolysin (*tdh*) production and one isolate was positive for the gene involved in both thermostable direct and thermostable direct related hemolysins (*trh*). Both isolates came from swimming ponds located in the Hilo area of the Island of Hawaii. In general, these hemolysins are associated with strains isolated from the feces of individuals infected with *V. parahaemolyticus* gastrointestinal
illness and are generally not present in environmental strains of this species. The two swimming ponds, one of which is thermally heated, from which these isolates were recovered are popular among swimmers. One possible explanation for the detection of hemolysin genes from these sites may be due to their accidental release by asymptomatically infected pond users. Another explanation may be that these ponds are being contaminated by cesspools in the neighboring area. Unlike on Oahu where sewer lines carry sewage to wastewater treatment plants, the Island of Hawaii heavily relies on

Table 6.3. The presence of hemolysin genes (tdh and trh) in *V. parahaemolyticus* strains isolated from Hawaii’s coastal waters.

<table>
<thead>
<tr>
<th>Isolated Site</th>
<th>No. of Isolates</th>
<th>No. <em>tdh</em> Positive (%)</th>
<th>No. <em>trh</em> Positive (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primary Coastal Beaches on Oahu</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Secondary Coastal Beaches on Oahu</td>
<td>3</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Coastal Harbors, Canals and Ponds on Oahu</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Harbors</td>
<td>6</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Canals</td>
<td>8</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Ponds</td>
<td>3</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Coastal Swimming Sites on Hawaii</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kona Area</td>
<td>6</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Hilo Area</td>
<td>10</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>High Temp., Low Salinity Ponds on Island of Hawaii</td>
<td>14</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Sediments</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Primary Coastal Beaches</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Secondary Coastal Beaches</td>
<td>106</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Human Sewage</td>
<td>4</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>TOTAL</td>
<td>160</td>
<td>2(1)</td>
<td>1(&lt;1)</td>
</tr>
</tbody>
</table>
cesspools for sewage collection. Based on data gathered in this study we know that *V. parahaemolyticus* can be sporadically detected in human sewage. Thus, it is likely that these pathogens may also be present in cesspools which can serve as a source of contamination of these bacteria into coastal ponds. Additionally, because one isolate came from a thermally heat pond (35°C) it may be likely that it came from a human source. Furthermore, due to Hawaii’s year-round warm water temperatures, strains that are released into environment have a better chance of survival as compared with temperate areas where cold water temperatures may lead to die of these strains.

### 6.5. SUMMARY AND CONCLUSIONS

Virulence determinants have historically been used to characterize strains of microorganisms as virulent or avirulent. Moreover, it is generally assumed that strains that are virulent are more likely to have come from a human source. However with some microorganisms, particularly those which are naturally found in the environment, the role of virulence determinants is not as clear. This is because the genes associated with virulence are generally not necessary in the day to day survival of these microorganisms, and only come into play when they have entered a suitable host. *Vibrio* spp. are generally associated with opportunistic infections, and strains, regardless of whether they harbor virulence genes, are capable of causing infections in humans. Thus the exact role virulence factors play in their pathogenicity has not been fully understood. However, categorizing species based on the presence of these factors can be a first step in assessing their virulence potential. The objective of this phase of the study was to characterize isolates of *V. cholerae*, *V. vulnificus* and *V. parahaemolyticus* isolates obtained from Hawaii’s coastal environments for the presence of known virulence determinants. We
hypothesized that these virulence determinants should be absent from our strains because it is known that environmental strains of these bacteria generally do not possess genes associated with virulence. In general, this was the trend we saw. Of the 114 isolates of *V. cholerae* analyzed, the gene associated with cholera toxin was detected in only one isolate. Similarly, with isolates of *V. parahaemolyticus* genes specific for hemolysin production was only detected in 1% of isolates. This is indicative that the majority of strains in or released into the environment may not contain factors associated with virulence. The majority (79%) of *V. vulnificus* isolates subtyped as B type or clinical strains. This has been observed in other studies where environmental strains of *V. vulnificus* were typed as type B 65% of the time. Seasonal variations have also been shown to affect 16S subtypes of *V. vulnificus* present in the environment. Type B strains have shown to be more prevalent when water temperatures are warm which may account for why these subtypes are more prevalent in Hawaii’s warm coastal waters. Though type B strains have similar 16S homology to clinical strains of *V. vulnificus*, their role in disease is not clear. Based on data gathered from this phase of the study we can say that our hypothesis was valid.
7.1. INTRODUCTION

The role of public health is to protect communities from adverse effects. Efforts are generally concerned with the population as a whole and not at an individual level. According to the USEPA, risks to populations are defined as the chance of harmful effects to human health or to ecological systems resulting from exposure to an environmental stressor. Risks in terms of water quality can include drowning, sun exposure or exposure to microbial pathogens. Pathogens including bacteria, protozoa and viruses can be discharged from untreated as well treated wastewater into streams and oceans. Standards based on fecal indicator bacteria have been established by the USEPA to safeguard the public against infections associated with pathogens present in human feces. However these standards do not apply to pathogens that are naturally present in aquatic environments.

*Vibrio* species are naturally occurring bacteria in marine environments and are known to cause opportunistic infections in humans. Infections can result either from ingesting contaminated seafood or through infection of wounds. Cholera due to *V. cholerae* infection is rare in the U.S. due to improvements in sanitation, but infections and death still occur in developing countries. *V. vulnificus* and *V. parahaemolyticus* lead to gastrointestinal infections associated with ingestion of contaminated seafood, and are more common in the U.S. *V. vulnificus* has also been implicated in wound infections associated with exposure of preexisting wounds to contaminated waters or with wounds
acquired from cleaning seafood. These infections have a large impact on public perception due to their gruesome nature and high mortality rate. These perceptions are often a result of the media attention these infections receive.

Microbial risk assessments are used to determine the likelihood that adverse human health effects will occur as a result of exposure to a particular pathogen. Basically, these assessments involve identifying the hazard, gathering data that link exposure to health effects and making recommendations based on the data gathered to estimate risk. Risks can be characterized in a quantitative or qualitative manner, depending upon the types and quality of data available, the use of risk estimates, and the tools that are available for the risk under assessment. Assessments can range from simple screening to complex, long-term studies.

Often times risk assessments are difficult to make because data linking exposure to health effects are not available. Data such as how many microbial cells are necessary to cause infection (dose response) are not always known. In the case of Vibrio spp., though all four species are capable of causing gastrointestinal infections, the infectious dose for these infections is generally high (>10⁶), thus, it is unlikely that recreational use of these waters will lead to such infections. On the other hand, all four species can also cause wound infections and the infectious dose for these types of infections has not yet been determined. Thus, it may be possible for even the presence of a small number of these bacteria to cause wound infections, particularly in individuals who have predisposing health conditions. When sufficient data is not available, quantitative risk assessments are difficult to make. Instead, qualitative assessments based on prevalence, exposure and severity can be used to determine the public health significance and
potential risk of pathogens. These risk estimates can then be used by public health professionals to make management decisions (such as allocation of funds) with regard to a particular pathogen. In the case of some pathogens, the only reasonable option available to managers is to communicate risk to the public regarding the use of recreational waters where pathogens are known to reside. Thus, qualitative risk assessments can be used to develop educational material for susceptible populations and sub-populations.

7.2. STUDY OBJECTIVES

Objective: To use *Vibrio* spp. prevalence data obtained from this study and CDC severity index data to determine the potential public health significance for infection by pathogenic *Vibrio* spp. when the general public uses the four categories of coastal waters for recreational purposes, and to recommend the posting of public notification levels at sites to communicate risk to the public.

Hypothesis: *Vibrio* spp. prevalence data can provide the first level of data that is currently not available to determine the potential public health significance to users of recreational waters.

7.3. EXPERIMENTAL DESIGN

The potential public health significance for each of the four pathogenic *Vibrio* spp. at each water quality category was determined based on two main factors (a prevalence index and a severity index) as previously described (section 3.10). Prevalence data gathered from this study was equated with the probability for infection, i.e. very high prevalence indicates very high probability for infection and a severity index was
developed for each *Vibrio* species based on published epidemiological data by the CDC between 1997 and 2008. Once the potential public health significance was determined for each *Vibrio* spp., it was determined what type of risk communication, via warning signs, was necessary to notify the public of the potential for infection from each species of vibrio.

### 7.4. RESULTS AND DISCUSSION

#### 7.4.1. Primary Coastal Beaches on Oahu (Table 7.1)

Primary coastal beaches consist of beaches that are popular among recreational users, thus exposure is considered to be very high at beaches in this category. *V*. *alginolyticus* was prevalent at 100% of the sites and was given a prevalence index of very high. This species, however, was given a low severity index based on CDC surveillance data collected between 1997 and 2008 (CDC, 2010a). Thus, there is no public health significance for *V*. *alginolyticus* infection. However, because this species had a high prevalence index at these sites, individual risk may be high in surfers and divers who receive cuts from coral which can then become infected. *V*. *vulnificus* has a high severity index because wound infections caused by this species progress rapidly and are generally fatal. *V*. *parahaemolyticus*, on the other hand, has a moderate severity index because this species can cause wound infections. However, its public health significance, in relation to *V*. *vulnificus*, can be considered low because infections are generally mild and non life threatening. Both species had a prevalence index of undetectable because they were not
detected in primary coastal beaches. Thus, there is no public health significance for *V. vulnificus* and *V. parahaemolyticus* in these types of waters. *V. cholerae* was not detected at any primary coastal beaches and was given a prevalence index of undetectable. This species has a low severity of illness index in the U.S. because there are relatively few infections and death is very rare. Thus, there is no public health significance for this species at these sites. Based on the low public health significance of the four pathogenic *Vibrio* spp. no signage is necessary at primary coastal beaches.

### 7.4.2. Secondary Coastal Beaches on Oahu (Table 7.2)

Sites belonging to the secondary beaches category are also used for recreational purposes. However, in relation to primary coastal beaches, swimmer exposure at these sites is considered to be moderate. These types of sites are characterized by lower salinity and higher turbidity than primary coastal beaches because these sites are often impacted by stream runoff. At these secondary coastal beaches (Table 7.2), *V. alginolyticus* was the most prevalent of the four species and has a prevalence index of very high. However, though prevalence of this species is very high, *V. alginolyticus* has a low severity index because the number of infections and deaths attributed to this species

<table>
<thead>
<tr>
<th>n = 20</th>
<th>Prevalence Index</th>
<th>Severity Index</th>
<th>Public Health Significance (Yes/No)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Vv</em></td>
<td>Undetectable</td>
<td>High</td>
<td>No</td>
</tr>
<tr>
<td><em>Vp</em></td>
<td>Undetectable</td>
<td>Moderate</td>
<td>No</td>
</tr>
<tr>
<td><em>Vc</em></td>
<td>Undetectable</td>
<td>Very Low</td>
<td>No</td>
</tr>
<tr>
<td><em>Va</em></td>
<td>Very High</td>
<td>Low</td>
<td>No</td>
</tr>
</tbody>
</table>

Table 7.1. The potential public health significance of *V. vulnificus* (*Vv*), *V. parahaemolyticus* (*Vp*), *V. cholerae* (*Vc*) and *V. alginolyticus* (*Va*) in primary coastal beaches based on a prevalence and severity index.
is relatively low. Thus, *V. alginolyticus* has no public health significance at these sites.

Individual risk, however, may be high because the prevalence of this species is very high, and infections are known to occur among surfers who receive cuts from coral which can then become infected. *V. vulnificus* has a moderate prevalence index and a high severity index, thus, this species has a public health significance at these sites. This is particularly true for individuals who have underlying health conditions, such as liver disease, because they are known to be more susceptible to illness than healthy individuals. In addition, because this species is prevalent at moderate levels and the infectious dose is not known only a small number of bacterial cells may be required to cause disease. Based on a low

Table 7.2. The potential public health significance of *V. vulnificus* (*Vv*), *V. parahaemolyticus* (*Vp*), *V. cholerae* (*Vc*) and *V. alginolyticus* (*Va*) in secondary coastal beaches based on a prevalence and severity index.

<table>
<thead>
<tr>
<th></th>
<th>Prevalence Index</th>
<th>Severity Index</th>
<th>Public Health Significance (Yes/No)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Vv</em></td>
<td>Moderate</td>
<td>High</td>
<td>Yes</td>
</tr>
<tr>
<td><em>Vp</em></td>
<td>Low</td>
<td>Moderate</td>
<td>No</td>
</tr>
<tr>
<td><em>Vc</em></td>
<td>Undetectable</td>
<td>Low</td>
<td>No</td>
</tr>
<tr>
<td><em>Va</em></td>
<td>Very High</td>
<td>Low</td>
<td>No</td>
</tr>
</tbody>
</table>

prevalence index and moderate severity index, *V. parahaemolyticus* does not pose a public health significance at secondary coastal beaches. Once again *V. cholerae* was not detected in secondary coastal beaches and was given a prevalence index of undetectable. This species has a low severity index in the U.S. because there are relatively few infections and death is very rare. Thus, there is no public health significance for this species at these sites. Beach sites that had a salinity range between 12 and 25ppt were sites that *V. vulnificus* was the most prevalent. Thus, when waters have a salinity within this range, the public should be warned of the potential risk for infection by this
pathogen. It is recommended that signs be posted at these sites warning users of the potential risk for infection by *V. vulnificus* particularly to individuals who have underlying health conditions or open wounds. Because salinity is an easily measureable parameter, city, state and private agencies are able to make this determination themselves relatively easily. Salinity measurements can be made from the mouth of streams, extending outwards, to determine the area where salinity is lowered, and therefore, the area at which the risk for infection may be the highest.

### 7.4.3. Coastal Harbors, Ponds and Canals on Oahu (Table 7.3)

Coastal harbor, pond and canal sites on Oahu are known to be highly impacted by land based discharge resulting in variable salinity. Human exposure at harbor, canal and pond sites generally does not occur, but low level exposure is known to occur. *V. alginolyticus* was once again the most prevalent of the four *Vibrio* species and had a prevalence index of very high (Table 7.3). However, because the number of illness and death attributed to this species is relatively low, it has a low severity index. Thus, *V. alginolyticus* has no public health significance at these sites. Individual risk, however, may be high because the prevalence of this species is very high, and infections are known to occur among surfers who receive cuts from coral which can then become infected. *V. cholerae* was not detected in any harbor, canal or pond sites and thus was given a prevalence index of undetectable. In addition, this species has a low severity index because infections and deaths in U.S. are relatively rare and mild. Thus, there is no public health significance for this species at these sites. At both harbor and canal sites, *V. parahaemolyticus* had both a moderate prevalence index and severity index while the prevalence index at canal sites was high. Though these sites are not approved for
swimming, low levels and accidental exposure by the public is known to occur.

However, because wound infections cause by this species is generally mild and self

Table 7.3. The potential public health significance of *V. vulnificus* (*Vv*), *V. parahaemolyticus* (*Vp*), *V. cholerae* (*Vc*) and *V. alginolyticus* (*Va*) in harbors, canals and ponds based on a prevalence and severity index.

<table>
<thead>
<tr>
<th></th>
<th>Prevalence Index</th>
<th>Severity Index</th>
<th>Public Health Significance (Yes/No)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Harbors</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(n = 11)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Vv</em></td>
<td>Very Low</td>
<td>High</td>
<td>No</td>
</tr>
<tr>
<td><em>Vp</em></td>
<td>Moderate</td>
<td>Moderate</td>
<td>No</td>
</tr>
<tr>
<td><em>Vc</em></td>
<td>Very Low</td>
<td>Low</td>
<td>No</td>
</tr>
<tr>
<td><em>Va</em></td>
<td>Very High</td>
<td>Low</td>
<td>No</td>
</tr>
<tr>
<td><strong>Canals</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(n = 7)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Vv</em></td>
<td>Moderate</td>
<td>High</td>
<td>Yes</td>
</tr>
<tr>
<td><em>Vp</em></td>
<td>High</td>
<td>Moderate</td>
<td>No</td>
</tr>
<tr>
<td><em>Vc</em></td>
<td>Very Low</td>
<td>Low</td>
<td>No</td>
</tr>
<tr>
<td><em>Va</em></td>
<td>Very High</td>
<td>Low</td>
<td>No</td>
</tr>
<tr>
<td><strong>Ponds</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(n = 5)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Vv</em></td>
<td>Low</td>
<td>High</td>
<td>No</td>
</tr>
<tr>
<td><em>Vp</em></td>
<td>Moderate</td>
<td>Moderate</td>
<td>No</td>
</tr>
<tr>
<td><em>Vc</em></td>
<td>Very Low</td>
<td>Low</td>
<td>No</td>
</tr>
<tr>
<td><em>Va</em></td>
<td>Very High</td>
<td>Low</td>
<td>No</td>
</tr>
</tbody>
</table>

limiting, *V. parahaemolyticus* does not pose a significant public health impact at harbor, pond and canal sites. The prevalence index of *V. vulnificus* at harbor and pond sites were very low and low, respectively. Though this species has a high severity index, their low prevalence at these sites indicates that this species poses no public health significance. At canal sites on the other hand, *V. vulnificus* had a prevalence index of moderate, which coupled with its high severity index indicates that this species does have a public health significance at these sites. It is recommended that warning signs be placed at canal sites
warning individuals to avoid coming into contact with these types of waters due to the possibility of infection by *V. vulnificus*. Individuals with open wounds and other immunocompromising health conditions should especially be careful to avoid these types of waters. This was illustrated in 2006 when an individual fell into one of these canal sites upon which he became infected with *V. vulnificus* and later succumbed to the infection. This individual was later found to have liver disease and cuts on his body at the time of exposure to the canal water. In the days prior to this individual falling into the canal, a 48 million gallon raw sewage leak was diverted into canal. *V. vulnificus* and *V. cholerae* have both been isolated from raw sewage thus during this sewage spill it is possible that large numbers of these bacteria were introduced into the canal. Because the spill continued for several days before it was stopped, introduced bacteria from the sewage, such as these pathogenic *Vibrio* spp., may have overtaken the bacteria naturally present in the canal making infection of anyone falling in more likely.

**7.4.4. Approved Swimming Sites on the Island of Hawaii (Table 7.4)**

Swimming ponds located on the Island of Hawaii are extensively used for recreational purposes because sandy beaches are not as widely present on this island as they are on Oahu. Thus, human exposure at all swimming sites located on the Island of Hawaii is considered high. *V. alginolyticus* had a prevalence index of high to very high levels in swimming ponds in the Kona and Hilo areas, including four low salinity, thermal ponds that were sampled. However, because this species causes mild, superficial infections, the severity index is considered low. Thus, *V. alginolyticus* does not have a public health significance at these sites. Individual risk, however, may be high because the prevalence of this species is very high, and infections are known to occur
among surfers and divers who receive cuts from coral which can then become infected.

*V. cholerae* was not detected at any of the coastal ponds and had a prevalence index of undetectable. This prevalence index, along with a low severity index, categorizes this species as having no major public health significance.

Table 7.4. The potential public health significance of *V. vulnificus* (*Vv*), *V. parahaemolyticus* (*Vp*), *V. cholerae* (*Vc*) and *V. alginolyticus* (*Va*) in swimming ponds on the Island of Hawaii based on a prevalence and severity index.

<table>
<thead>
<tr>
<th></th>
<th>Prevalence Index</th>
<th>Severity Index</th>
<th>Public Health Significance (Yes/No)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Kona Area (n = 9)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Vv</em></td>
<td>Low</td>
<td>High</td>
<td>No</td>
</tr>
<tr>
<td><em>Vp</em></td>
<td>Low</td>
<td>Moderate</td>
<td>No</td>
</tr>
<tr>
<td><em>Vc</em></td>
<td>Very Low</td>
<td>Low</td>
<td>No</td>
</tr>
<tr>
<td><em>Va</em></td>
<td>High</td>
<td>Low</td>
<td>No</td>
</tr>
<tr>
<td><strong>Hilo Area (n = 7)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Vv</em></td>
<td>Moderate</td>
<td>High</td>
<td>Yes</td>
</tr>
<tr>
<td><em>Vp</em></td>
<td>Moderate</td>
<td>Moderate</td>
<td>No</td>
</tr>
<tr>
<td><em>Vc</em></td>
<td>Very Low</td>
<td>Low</td>
<td>No</td>
</tr>
<tr>
<td><em>Va</em></td>
<td>Very High</td>
<td>Low</td>
<td>No</td>
</tr>
<tr>
<td><strong>Low Salinity, Thermal Ponds (n = 4)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Vv</em></td>
<td>Very High</td>
<td>High</td>
<td>Yes</td>
</tr>
<tr>
<td><em>Vp</em></td>
<td>High</td>
<td>Moderate</td>
<td>No</td>
</tr>
<tr>
<td><em>Vc</em></td>
<td>Very Low</td>
<td>Low</td>
<td>No</td>
</tr>
<tr>
<td><em>Va</em></td>
<td>Very High</td>
<td>Low</td>
<td>No</td>
</tr>
</tbody>
</table>

In swimming ponds located in the Kona area of the Island of Hawaii, both *V. vulnificus* and *V. parahaemolyticus* had a low prevalence index. Thus, though *V. vulnificus* and *V. parahaemolyticus* have a severity index of high and moderate, respectively, there is no major public health significance associated with both species at these coastal sites because of their low prevalence. At swimming ponds in the Hilo area
both *V. vulnificus* and *V. parahaemolyticus* had a prevalence index of moderate and a severity index of high and moderate, respectively. Thus, *V. vulnificus* is considered to have a public health significance at these sites, while *V. parahaemolyticus* does not because wound infections associated with this species are mild and self limiting.

Low salinity, thermal ponds are unique to the Island of Hawaii. At these ponds, *V. vulnificus* had a prevalence index of very high, which along with a high severity index indicates that this species does pose a public health significance. These types of ponds are popular among swimmers, particularly with tourists, and infections and deaths related to *V. vulnificus* have occurred as a result of swimming in these types of ponds. *V. parahaemolyticus* had a high prevalence index and a moderate severity index at these thermal ponds. However, because this species generally causes mild, self limiting infections, it does not pose a public health significance. Thus, it is recommended that warning signs be placed at low salinity swimming ponds and low salinity thermal ponds in the Hilo area of the Island of Hawaii warning individuals to avoid coming into contact with these types of waters due to the possibility of infection by *V. vulnificus*. Individuals with open wounds and other immunocompromising health conditions should especially be careful to avoid these types of waters. Currently similar types of signs have been posted at some of these ponds due to law suits brought on by family members who have lost loved ones to *V. vulnificus* infections. However, though these types of infections are not always publically documented, infections continue to occur. Thus, it is important that the potential risk is communicated effectively to the public.

This phase of the study was able to provide basic, preliminary data on the potential public health significance of *V. vulnificus, V. cholerae, V. parahaemolyticus* and
*V. alginolyticus* in four water quality categories in Hawaii. These types of assessments, however, are more complex and involve a variety of factors. For example, with bacterial pathogens, and opportunistic pathogens in particular, it is not certain whether all strains found in the environment are capable of causing infection in humans. Most *V. vulnificus* strains are considered to be virulent but there may be strains that are more virulent than others. Studies with *V. parahaemolyticus* have shown that some strains possess virulence genes that make them more virulent than strains that do not possess these genes. Strains isolated from the environment generally lack these genes, and it is assumed that these strains are not be capable of causing infection in humans. It is thought that this is the case because these virulent strains are outnumbered by avirulent strains in the environment, and culture methods are only capable of detecting strains at the highest concentrations. However, if these virulent strains gain entrance into a human host, they may possess certain factors that enable them to outcompete other bacteria to establish infection. Host susceptibility is another factor that affect whether disease results when a host encounters a pathogen. This is particularly true for pathogens, like *Vibrio* spp., that cause opportunistic infections. It is known that individuals with chronic illnesses like hepatitis, liver disease, and AIDS are more susceptible to these types of infections. Thus, the risk for *Vibrio* spp. infections can be very high for certain segments of the population as compared to the general population. Furthermore, marine sediments are known to harbor high densities of bacteria which can then be a source of these bacteria into the water column. Bacteria found in these sediments may differ, in terms of possessing virulence factors, from those of similar species of bacteria found in the water column.
This study provides a basic method of determining the public health significance of pathogenic *Vibrio* spp. based on a prevalence and severity index, and does not take into account factors such as the contribution of the four pathogenic *Vibrio* species present in sediment biofilms. It also does not take into account unknown factors such as infectious dose which is currently unknown for wound infections caused by the four *Vibrio* pathogens. However, this study may provide the initial data needed to conduct a more thorough assessment which may eventually lead to the development of water quality standards for *Vibrio* spp. This may be likely in the future when the gaps in our knowledge about these pathogens are filled.

### 7.5. SUMMARY AND CONCLUSIONS

The major function of public health is to protect communities from adverse effects. In relation to water quality, standards based on fecal indicator bacteria have been set by the USEPA to safeguard the public from fecal/sewage borne pathogens. These standards however are not effective in protecting populations from bacteria, such as *Vibrio* species, that occur naturally in aquatic environments. These bacteria are generally associated with opportunistic infections in humans. Risk assessments are used to determine the likelihood of an adverse health effect occurring as a result of exposure to a particular hazard (i.e. pathogen). Epidemiological studies offer the best method of showing a causal relationship between human exposure to a pathogen and disease. However these types of studies can be costly and not useful when working with rare disease outcomes. Quantitative Microbial Risk Assessment (QMRA) is an alternative to epidemiological studies. Unlike epidemiological studies, QMRA can provide a direct measurement of pathogens and their association with disease. However, often times the
necessary data and resources needed for QMRA (such as dose response data) is not available. In such cases risk can be determined by the use of a severity of illness index. These indices can include such data as duration of illness, case fatalities rates, prevalence to determine risk and can be either quantitative or qualitative. The objective of this final phase of study was to use *Vibrio* spp. prevalence data obtained from this study to determine the potential public health significance for infection by pathogenic *Vibrio* spp. when the general public uses the four categories of coastal waters for recreational purposes, and to recommend the posting of public notification levels at sites to communicate risk to the public. We hypothesized that this *Vibrio* spp. prevalence data can provide the first level of data that is currently not available to determine the potential public health significance to users of recreational waters. Based on two factors, a prevalence index and a severity of illness index, it was determined whether each of the four pathogenic *Vibrio* spp. poses a public health significance at each of the four water quality categories, and then what the public notification level would be.

As hypothesized, prevalence data gathered from this study was able to provide a basic assessment of the potential public health significance pathogenic *Vibrio* species have on humans who use Hawaii’s coastal waters for recreational purposes. Based on this assessment, it is recommended that signs be posted at secondary coastal beaches advising users that, due to impact by stream runoff and the resulting lowered salinity, there is no guarantee as to the safety of the water, and that individuals who have underlying health conditions or open wounds should be particularly discouraged from recreating in these types of waters, particularly at times when stream inputs at the beaches are at their highest. It is also recommended that signs be posted at canal sites
and low salinity swimming ponds and low salinity thermal ponds in the Hilo area of the Island of Hawaii warning individuals to avoid coming into contact with these types of waters due to the possibility of infection by *V. vulnificus*. It is also known that infections by these *Vibrio* spp. in immunocompromised individuals are generally more frequent and severe. They are known to cause particularly severe infections in individuals with cirrhosis, hepatitis and cancer of the liver. Thus, because no standard is currently available for *Vibrio* species, the most effective way of preventing illness and death due to recreational water use is to effectively communicate risk to the public. *Vibrio* spp. prevalence data gathered from this study can provide the first level of data that is currently not available to determine the potential public health significance to users of recreational waters which can then be used to communicate risk to the public.

Proper signage language to communicate risk to people who choose to enter bodies of environmental waters for various purposes is a difficult task. Two examples are discussed:

*Signage for violation of current recreational water quality standards.* Warning signs can be posted to close the beach for swimming when the concentrations of fecal indicator bacteria exceed the water quality standards.

The basic problem with these warning signs is that the water quality measurements were made the previous day and the beach is closed on the following day because the test results are read 24 hours after the sample is processed. Therefore, the day the water quality exceeded the standard, the beaches were approved for swimming. Information on the water quality on the day the beach has been closed is not available to the public. Moreover, most of the monitoring data has shown that the when a body of
water exceeded the water quality standards on a given day, that water quality data cannot be reliably extrapolated to indicate the quality of water at that beach site for the following day. In some cases, exceedance of fecal indicator concentrations have been shown to meet water quality standards within a few minutes to a few hours after the initial water sample was taken.

*Signage for leptospirosis transmission for freshwater sites in Hawai‘i.* The Hawaii Department of Health (HDOH) has determined that it cannot measure for presence or prevalence of pathogenic *Leptospria* sp. in any of the stream water sites. However, because there has been a direct association with occupational or recreational exposure to freshwater sites in Hawai‘i and transmission of leptospirosis, the HDOH has adopted a strategy of posting a warning sign at all fresh water sites in the state of Hawaii. With this strategy, everyone is considered equally susceptible and every freshwater site is considered a health risk for transmission of pathogenic *Leptospria* sp. The warning signs reads as follows:

Warning! Leptospirosis Health Hazard. Fresh water streams and mud possibly polluted with bacteria. Swim or hike at your own risk. For more information call the Hawaii Department of Health.

*Proposed signage for risk related to infection by V. vulnificus.* Currently certain swimming sites on the Island of Hawai‘i have signs posted warning swimmers of possible bacterial infections. On Oahu, at sites where *V. vulnificus* is prevalent, the following signage is recommended:

Notice: Bacterial testing indicates that pathogenic *Vibrio* spp. may be present at this site. Individuals with open wounds should avoid coming into contact with water due to the possibility of infection by these bacteria. Enter at your own risk.
CHAPTER 8
SUMMARY, CONCLUSIONS AND FUTURE DIRECTION

8.1. PROJECT RATIONALE AND PROJECT GOAL

The genus *Vibrio* is characterized by gram-negative, comma shaped bacteria that contain one or more flagella. Members of the genus *Vibrio* are ubiquitous and are widely distributed in aquatic environments where they fulfill a major ecological role in the cycling of organic and inorganic compounds in the water. In addition to their role in nature, certain species of *Vibrio* are known to cause opportunistic infections in both aquatic animals as well as humans. A small number of *Vibrio* species can cause opportunistic infections in humans, resulting in mainly wound or gastrointestinal illness. Though most infections in humans are generally mild and self limiting, high mortality rates in immunocompromised individuals are known to occur. The majority of human infections are attributed to four species of *Vibrio*: *V. cholerae*, *V. vulnificus*, *V. parahaemolyticus* and *V. alginolyticus*. These four species cause a variety of infections from mild gastrointestinal infection to septicemia and death. Most of what we know about the ecology of *Vibrio* species are from studies conducted in temperate regions. These studies have revealed that the two most significant factors that affect the ecology of *Vibrio* species are water temperature and salinity. As water temperatures drop below $15^\circ$C this species becomes undetectable and when water temperatures increase above $15^\circ$C they become detectable once again. Salinity is a second important factor that has been shown to influence the abundance of different *Vibrio* species. Therefore, salinity requirements can and have been used to characterize the different *Vibrio* species.

Information on the prevalence and ecology of *Vibrio* species in tropical areas, such as Hawaii, is limited. Up to now, there has been no study conducted in Hawaii to
determine the prevalence of these pathogens in our coastal waters. Thus, with this study, we hope to answer questions about the prevalence of pathogenic *Vibrio* species in Hawaii’s coastal waters and their potential public health significance to users of our coastal waters for recreational purposes. The major goal of this study was to determine the prevalence of the four human pathogenic *Vibrio* species (*V. cholerae*, *V. vulnificus*, *V. parahaemolyticus*, *V. alginolyticus*) in coastal water environments of Hawaii and to determine the public health significance these pathogens have to people who use these coastal water for recreational purposes.

### 8.2. PROJECT EXPERIMENTAL DESIGN

The experimental design for this experiment involved the collection and analysis of various types of water samples. Sampling sites on Oahu included those from beaches impacted and not impacted by land run-off and confined coastal waters (harbors, canals, ponds). Sediments from coastal beaches on Oahu and human sewage were also analyzed for the presence of the four *Vibrio* pathogens. This was to determine if sediments and sewage could be possible sources of contamination of these pathogens into our coastal waters. Sampling sites on the Island of Hawaii included coastal ponds located in the Kona and Hilo areas as well thermal ponds located in Hilo. The prevalence of the four pathogenic *Vibrio* species was determined based on growth on CHROMagar Vibrio, presumptive identification using biochemical testing and PCR confirmation. Confirmed isolates were further characterized for virulence determinants using PCR.
8.3. MAJOR FINDINGS OF THIS STUDY

The first goal of this study was to establish reliable and feasible methods to recover the four human pathogenic *Vibrio* species (*V. cholerae*, *V. vulnificus*, *V. parahaemolyticus*, *V. alginolyticus*) from coastal waters of Hawaii and to characterize their virulence potential. This study relied on the use of a relatively new agar, CV agar, in combination with FDA recommended biochemical tests to characterize *Vibrio* spp. recovered from Hawaii’s coastal waters. Previously, TCBS agar had been the media of choice for the isolation of *Vibrio* spp., but initial discrimination of species on this agar has been difficult. This is because TCBS relies on the fermentation of sucrose to distinguish between *Vibrio* spp., and samples containing sucrose positive species tend to overwhelm the plate, masking sucrose negative colonies. CV agar, on the other hand, distinguishes *Vibrio* spp. based on color, making the initial discrimination of species much easier. Current, approved methods for *Vibrio* spp. detection still rely on culture and are thus time consuming, however, the use of a media such as CV agar can make the initial screening more efficient, saving both time and resources.

The second and third goals of this study were to apply the new method to determine the prevalence of these four human pathogenic vibrio in the major classes or categories of coastal waters, and to determine how selected environmental conditions (salinity, temperature, turbidity, nutrient level) affect the prevalence of the four pathogenic *Vibrio* spp. in Hawaii’s coastal waters. We found that *V. vulnificus* and *V. parahaemolyticus* were prevalent in low salinity sites that were impacted by land run-off but not detectable in high salinity, non-impacted swimming sites. *V. alginolyticus* was prevalent in all sites regardless of salinity. Though this species is generally associated
with high salinity waters, nutrients brought in by land run-off can overcome salinity barriers and stimulate *Vibrio* species growth. Furthermore, *V. vulnificus* and *V. parahaemolyticus* were prevalent at low salinity swimming ponds on the Island of Hawaii, indicating once again that salinity has a strong influence on the prevalence of these two species. In addition to low salinity, high water temperature also had an impact on the concentration of the four *Vibrio* spp. pathogens. Though the overall counts of bacteria were lower in these ponds as compared to non-thermally heated ponds, *V. alginolyticus*, *V. vulnificus* and *V. parahaemolyticus* were prevalent in these thermal ponds. Thus, these high temperature, low salinity ponds can select for these three *Vibrio* pathogens. Furthermore, there has been past evidence of infection and death due to *V. vulnificus* associated with the use of these ponds. Isolates recovered from these thermal ponds may potentially be more virulent as they have been adapted to survival at temperatures similar to that of human body temperature. *V. cholerae* was not recovered in either impacted or non-impacted sites. Studies have shown that this species is typically found in close association with chitinous organisms, such as zooplankton, during times of environmental stress. Therefore it may be possible that because these sites had salinities above those which are tolerable by *V. cholerae* they may be more likely to be attached to zooplankton than in the water column.

The fourth goal of this study was to determine if biofilm growth in marine environment or sewage can serve as external sources of *Vibrio* spp. recovered from water samples obtained from coastal water sites. Data from this study showed that the prevalence of pathogenic *Vibrio* species in sediments followed a similar trend to what was seen with coastal beach samples. *V. alginolyticus* was prevalent in both primary and
secondary beach sediment while *V. vulnificus* and *V. parahaemolyticus* were only prevalent in secondary beach sediment. These sites have lower salinity and are influenced by land run-off and these two species were also prevalent in the water columns of these types of sites. Based on data from this study it is apparent that sediments from secondary coastal waters can serve as a source of pathogenic *Vibrio* species into the water column.

Data from this study also showed that *V. vulnificus* and *V. parahaemolyticus* were sporadically present in raw and primary treated sewage from three different wastewater treatment plants. *V. cholerae* on the other hand was consistently recovered in raw and primary treated sewage from all three treatment plants. Based on this data it is apparent that sporadic shedding of *V. vulnificus* and *V. parahaemolyticus* is occurring within the population, while *V. cholerae* is being consistently shed by asymptomatic carriers. Thus, accidental release of untreated sewage can introduce these pathogens into coastal waters which may lead to public health consequences to users of these waters.

Of the 114 isolates of *V. cholerae* analyzed, the gene associated with cholera toxin was detected in only one isolate. Similarly, with isolates of *V. parahaemolyticus* genes specific for hemolysin production was only detected in 1% of isolates. This is indicative that the majority of strains in or released into the environment may not contain factors associated with virulence. The majority (74%) of *V. vulnificus* isolates subtyped as B type or clinical strains. This has been observed in other studies where environmental strains of *V. vulnificus* were typed as type B 65% of the time. Seasonal variations have also been shown to affect 16S subtypes of *V. vulnificus* present in the environment. Type B strains have shown to be more prevalent when water temperatures
are warm which may account for why these subtypes are more prevalent in Hawaii’s warm coastal waters. Though type B strains have similar 16S homology to clinical strains of *V. vulnificus*, their role in disease is not clear.

The fifth goal was to use the prevalence data obtained from this study to determine the relative public health significance of the four *Vibrio* spp. when people choose to use the four categories of coastal waters for recreational purposes, and to determine if warning signs were warranted at these sites. Data from this study revealed that though *V. alginolyticus* was the most prevalent *Vibrio* species detected it did not pose a significant public health threat. This is because infections related to this species are relatively mild and self limiting. It was also determined that *V. cholerae* did not pose a significant threat to public health as it was not detected in any of our coastal waters. However, data from this study also revealed that *V. cholerae* was present in raw sewage. Thus, accidental sewage spills into coastal waters may increase the public health significance of these pathogens. In contrast to *V. alginolyticus* and *V. cholerae*, *V. vulnificus* and *V. parahaemolyticus* can cause wound infections. However it was determined that *V. parahaemolyticus* does not pose a significant public health significance because infections attributed to this species are generally mild and not life threatening. *V. vulnificus* on the other hand is capable of causing severe wound infections which can rapidly lead to death. Thus, this species does pose a public health significance, and it is therefore recommended that signs be placed in areas where this species is prevalent warning users of the potential for infection. As hypothesized, the data gathered from this study was able to provide a basic assessment of the potential public health significance pathogenic *Vibrio* spp. have on humans who use Hawaii’s
coastal waters for recreational purposes. Based on this data we were able to determine if and when warning signs would be warranted to notify the public of the potential risk for infection.

By fulfilling the above objectives, this study was successfully able to meet its goals of: determining the prevalence of the four human pathogenic *Vibrio* species in four categories of coastal water environments in Hawaii, and determining the public health significance these pathogens have to the public who use these coastal waters for recreational purposes. Thus, data gathered from this study may provide some answers that were lacking regarding the distribution of these pathogens in tropical areas such as Hawaii.

### 8.4. Future Direction

1. We would like to continue our sampling of coastal beaches on Oahu and the Island of Hawaii. Several of the water quality categories contained less than 10 sampling sites. Samples of canal and pond sites on Oahu were limited as were samples from thermal ponds. Thus, in order to make our conclusions more robust, more sites need to be sampled. The thermal ponds are particularly interesting due to the prevalence of *V. vulnificus* and *V. parahaemolyticus* strains that can withstand high temperatures. These sites are popular among tourists and the potential for infection may be high.

2. One major limitation of this study was the limited sample volume that could be processed with membrane filtration and growth on media. Due to high concentrations of bacteria in samples, they had to be diluted, thus potentially also diluting target bacteria. An alternative to culture is the use of molecular methods such as PCR. PCR
can potentially allow for the processing of larger sample volumes through concentration methods. However, the interpretation of PCR results for environmental waters in terms of public health is still not approved. But, PCR data may perhaps be used to supplement our culture data.

3. This study provides data, which was lacking, regarding the prevalence of pathogenic *Vibrio* species in Hawaii’s diverse coastal water environment. Furthermore, it provides a preliminary assessment of the potential public health consequences these pathogenic *Vibrio* species have to users of Hawaii’s coastal waters. This data can now be used to narrow our focus to specific sites to determine the effect additional environmental factors (nutrients, sunlight) have on the prevalence of these bacteria. In addition, repeat sampling of each site would be necessary to make the data more scientifically sound.

4. Another habitat that would be important to sample are zooplankton. *Vibrio* species, particularly *V. cholerae*, are known to be attached to the surfaces of these organisms. It is also known that this attachment occurs during times of environmental stress (high water temperature, low nutrients). Thus, one reason we may not have detected *V. cholerae* in the water column may have been because they are more prevalent on surface of zooplankton.

5. Finally, because we were able to consistently detect *V. cholerae* in sewage influent, it would be interesting to test human fecal samples for this bacteria. It is apparent that there are many asymptomatic infections occurring within Hawaii’s population, and an epidemiological study to track down the source of these infections may be interesting.
APPENDIX A

MAPS OF SAMPLING SITES ON OAHU AND THE ISLAND OF HAWAII

Figure A-1. Primary coastal beach sites located on Oahu.

Figure A-2. Secondary coastal beach sites located on Oahu.
Figure A-3. Non-swimming coastal sites located on Oahu.
APPENDIX B

RAW PHYSICAL AND MICROBIAL DATA OF THE FOUR WATER QUALITY CATEGORIES ON OAHU AND THE ISLAND OF HAWAII

Table B-1. Physical (salinity and turbidity) and microbial (total marine and vibrio) data of primary coastal beaches on Oahu.

<table>
<thead>
<tr>
<th>Beach Name</th>
<th>Label on Map</th>
<th>Salinity (ppt)</th>
<th>Turbidity (NTU)</th>
<th>Marine Agar (CFU/100ml)</th>
<th>TCBS (CFU/100ml)</th>
<th>CV (CFU/100ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ala Moana Beach</td>
<td>A1</td>
<td>36</td>
<td>3.410</td>
<td>1.1 x 10^4</td>
<td>3.6 x 10^3</td>
<td>4.1 x 10^3</td>
</tr>
<tr>
<td>Magic Island</td>
<td>A2</td>
<td>36</td>
<td>1.326</td>
<td>6.2 x 10^4</td>
<td>4.2 x 10^3</td>
<td>1.2 x 10^3</td>
</tr>
<tr>
<td>Waikiki Beach</td>
<td>A3</td>
<td>36</td>
<td>4.800</td>
<td>4.2 x 10^4</td>
<td>1.1 x 10^3</td>
<td>8.4 x 10^2</td>
</tr>
<tr>
<td>San Souci Beach</td>
<td>A4</td>
<td>35</td>
<td>3.190</td>
<td>4.1 x 10^4</td>
<td>6.8 x 10^3</td>
<td>5.6 x 10^3</td>
</tr>
<tr>
<td>Kuhio Beach</td>
<td>A5</td>
<td>35</td>
<td>2.850</td>
<td>6.6 x 10^4</td>
<td>3.3 x 10^3</td>
<td>3.8 x 10^3</td>
</tr>
<tr>
<td>Oneawa Beach</td>
<td>A6</td>
<td>35</td>
<td>2.032</td>
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<td>6.6 x 10^3</td>
<td>8.2 x 10^4</td>
</tr>
<tr>
<td>Lanikai Beach</td>
<td>A7</td>
<td>36</td>
<td>2.659</td>
<td>1.6 x 10^4</td>
<td>6.8 x 10^3</td>
<td>8.9 x 10^3</td>
</tr>
<tr>
<td>Kualoa Beach Park - 1st Bathroom</td>
<td>A8</td>
<td>35</td>
<td>3.021</td>
<td>1.7 x 10^4</td>
<td>8.0 x 10^3</td>
<td>8.5 x 10^3</td>
</tr>
<tr>
<td>Sandy Beach</td>
<td>A9</td>
<td>35</td>
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<td>Laie Beach Park</td>
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Table B-2. Physical (salinity and turbidity) and microbial (total marine and vibrio) data of secondary coastal beaches on Oahu.

<table>
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<th>Label on Map</th>
<th>Salinity (ppt)</th>
<th>Turbidity (NTU)</th>
<th>Marine Agar (CFU/100ml)</th>
<th>TCBS (CFU/100ml)</th>
<th>CV (CFU/100ml)</th>
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<tbody>
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<td>Kawaikui Beach Park B1</td>
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<td>9.8 x 10^5</td>
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<td>6.5 x 10^4</td>
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Table B-3. Physical (salinity and turbidity) and microbial (total marine and vibrio) data of non-swimming coastal sites (harbors, canals, ponds) on Oahu.

<table>
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<tr>
<th>Label on Map</th>
<th>HARBORS</th>
<th>Salinity (ppt)</th>
<th>Turbidity (NTU)</th>
<th>Marine Agar (CFU/100ml)</th>
<th>TCBS (CFU/100ml)</th>
<th>CV (CFU/100ml)</th>
</tr>
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<tbody>
<tr>
<td>Ala Wai Yacht Harbor</td>
<td>C1</td>
<td>32</td>
<td>3.120</td>
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<td>$1.5 \times 10^4$</td>
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<tr>
<td>Pearl Harbor Each Loch</td>
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<td>$4.0 \times 10^4$</td>
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<tr>
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<td>$6.6 \times 10^3$</td>
<td>$6.3 \times 10^3$</td>
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<td>Pearl Harbor West Loch</td>
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<td>33</td>
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<td>$7.6 \times 10^3$</td>
<td>$2.5 \times 10^4$</td>
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<tr>
<td>Heeia Boat Harbor</td>
<td>C5</td>
<td>32</td>
<td>17.500</td>
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<td>$2.1 \times 10^4$</td>
<td>$5.2 \times 10^4$</td>
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<td>$1.1 \times 10^4$</td>
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<td>Honolulu Harbor</td>
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<td>$5.4 \times 10^3$</td>
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<td>Koolina Marina</td>
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<td>Ala Wai Canal</td>
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<td>PONDS</td>
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<tr>
<td>Pond at Ala Moana Beach</td>
<td>E1</td>
<td>33</td>
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Table B-4. Physical (salinity and turbidity) and microbial (total marine and vibrio) data of coastal swimming ponds on the Island of Hawaii.

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<th>Label on Map</th>
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<th>Turbidity (NTU)</th>
<th>Marine Agar (CFU/100ml)</th>
<th>TCBS (CFU/100ml)</th>
<th>CV (CFU/100ml)</th>
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<td>$8.4 \times 10^4$</td>
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</tbody>
</table>
APPENDIX C

PRESENCE OF PATHOGENIC *VIBRIO* SPECIES IN THE FOUR WATER QUALITY CATEGORIES ON OAHU AND THE ISLAND OF HAWAII

Table C-1. The presence/absence of *V. vulnificus*, *V. cholerae*, *V. parahaemolyticus* and *V. alginolyticus* in primary coastal beaches on Oahu.

<table>
<thead>
<tr>
<th>Beach</th>
<th><em>V. vulnificus</em></th>
<th><em>V. cholerae</em></th>
<th><em>V. parahaemolyticus</em></th>
<th><em>V. alginolyticus</em></th>
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<td>Lanikai Beach</td>
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Table C-2. The presence/absence of *V. vulnificus*, *V. cholerae*, *V. parahaemolyticus* and *V. alginolyticus* in secondary coastal beaches on Oahu.

<table>
<thead>
<tr>
<th></th>
<th><em>V. vulnificus</em></th>
<th><em>V. cholerae</em></th>
<th><em>V. parahaemolyticus</em></th>
<th><em>V. alginolyticus</em></th>
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<tbody>
<tr>
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<td>Waiahole Beach Park</td>
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<td>Kaneohe Bay at Heeia Stream</td>
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<td>Kaneohe Bay at Kaneohe Stream</td>
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<td>Kalaeoio Beach Park</td>
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<td>Punaluu Beach Park</td>
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<td>Kuliouou Beach Park</td>
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Table C-3. The presence/absence of *V. vulnificus*, *V. cholerae*, *V. parahaemolyticus* and *V. alginolyticus* in non-swimming coastal sites (harbors, canals, ponds) on Oahu.

<table>
<thead>
<tr>
<th>Location</th>
<th><em>V. vulnificus</em></th>
<th><em>V. cholerae</em></th>
<th><em>V. parahaemolyticus</em></th>
<th><em>V. alginolyticus</em></th>
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<td><strong>HARBORS</strong></td>
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<td>Pearl Harbor Each Loch</td>
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<tr>
<td>Pearl Harbor Middle Loch</td>
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<td>Pearl Harbor West Loch</td>
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<td>Heeia Boat Harbor</td>
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<tr>
<td>Fisherman’s Wharf</td>
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<tr>
<td>Honolulu Harbor</td>
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<tr>
<td>Koolina Marina</td>
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<td>Barber’s Point Harbor</td>
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<td>Keehi Boat Harbor</td>
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<td>Stream near Down-to-Earth (Kailua)</td>
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Table C-4. The presence/absence of *V. vulnificus*, *V. cholerae*, *V. parahaemolyticus* and *V. alginolyticus* in coastal swimming ponds on the Island of Hawaii.

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<th></th>
<th><em>V. vulnificus</em></th>
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<th><em>V. parahaemolyticus</em></th>
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<td><strong>HILO AREA PONDS</strong></td>
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<td>Goya Pond</td>
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<td>Main Pond in MLCD Tide Pools</td>
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<td>Outer Lagoon in MLCD Tide Pools</td>
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<td>Inner Lagoon in MLCD Tide Pools</td>
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Austin, B., D. Austin, R. Sutherland, F. Thompson and J. Swings. 2005. Pathogenicity of vibrios to rainbow trout (Oncorhynchus mykiss, Walbaum) and Artemia nauplii. Environmental Microbiology 7(9):1488-1495.


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