# INVESTIGATION INTO PATHOGENIC VIBIO PARAHAEMOLYTICUS DYNAMICS AND VIRULENCE DETERMINANTS FROM HAWAIIAN WATERS

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I certify that I have read this thesis and that, in my opinion, it is satisfactory in the scope and quality as a thesis for the degree of Bachelor of Science in Global Environmental Science.

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# ABSTRACT

The pathogenic gram-negative halophilic bacterium, *Vibrio parahaemolyticus*, is a water-borne human pathogen indigenous to coastal marine and estuarine environments. When introduced to a human host, either by the consumption of raw or undercooked shellfish, or by exposure to an open wound, the pathogen can cause gastroenteritis, tissue infection, and, in some cases, septicemia. The risk of human infection is expected to be directly associated to the abundance of pathogenic *V. parahaemolyticus*. To evaluate how environmental conditions may influence the abundance of this pathogen I investigated the spatial, temporal, and environmental prevalence of *Vibrio parahaemolyticus* in the Ala Wai Canal and surrounding waters of Honolulu, Hawai'i. Strains of *V. parahaemolyticus* were isolated on selective media along an environmental gradient and at frequencies ranging from months to hours. Putative *V. parahaemolyticus* isolates were identified using chromogenic media. Their identities were later confirmed using molecular methods, and they were analyzed for the presence of known virulence-associated genes. The species-specific(*tlh*) gene was found in 79% of the putative *V. parahaemolyticus* isolates, but none of these were positive for a virulence-associated gene (*tdh*). There was a positive correlation between total *V. parahaemolyticus* abundance and salinity, but only in the range from 2.5-19 ppt. No significant correlation was found between temperature and *V. parahaemolyticus* abundance. Highest *V. parahaemolyticus* densities were usually found at the head of the canal and at the mouths of the two streams feeding into the canal (stations 1-6, 9 and 12). This information may prove useful for modeling pathogen dynamics in tropical coastal environments.

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# CHAPTER 1

# INTRODUCTION AND BACKGROUND

The genus *Vibrio* includes more than sixty-five species, at least twelve of which are pathogenic to humans or have been associated with foodborne diseases (Chakraborty, et al., 2000). Among these species, *Vibrio parahaemolyticus* resides in many of the world's estuarine waters and is a leading cause of foodborne gastroenteritis. *Vibrio parahaemolyticus* is a halophilic bacterium and was first identified in the 1950's as a cause of foodborne illnesses in Japan. More recently this *vibrio* has been identified with wound infections and septicemia in susceptible hosts (Morris and Black, 1985). Although *Vibrio parahaemolyticus* is currently recognized as one of the major causal agents of seafood related gastroenteritis, not all strains of this species are considered to be truly pathogenic (Mead, et al, 1999). Annually, *Vibrio*-related illnesses account for over eight thousand cases of gastroenteritis in the USA. Specific to the *Vibrio parahaemolyticus* bacterium, it is estimated that there are at least 4500 cases of infection occurring each year in the United States. However, this may be an underestimate, because the surveillance of the Centers for Disease Control and Prevention is hampered by underreporting (National Center for Zoonotic, Vector-Borne, and Entreric Diseases Online, 2008). The majority of outbreaks of gastroenteritis in the United States have been related to contact with and consumption of raw or undercooked shellfish (Bean, et al., 1998).

In the USA, outbreaks were first identified in 1971 during which 320 patients suffered from gastroenteritis due to food poisoning (Molero, 1982, also Dadisman, et al, 1972). *Vibrio parahaemolyticus* has also been isolated from seawater and seafood of the Hawaiian Archipelago since the early 1960's (Yasunaga, 1965).

### *Clinical Disease*

*Vibrio parahaemolyticus* causes three major syndromes of clinical illness in humans with the most common syndrome being gastroenteritis, followed by wound infections and septicemia (Morris and Black, 1985; Daniels, et al, 2000). When ingested, *Vibrio parahaemolyticus* causes watery diarrhea, often with abdominal cramping, nausea, vomiting, fever and chills. Usually these symptoms occur within twenty-four hours of ingestion. Illness is usually self-limited and lasts up three days. Life threatening complications are rare and transpire more commonly in people with weakened immune systems. *Vibrio parahaemolyticus* can also cause an infection of the skin when an open wound is exposed to warm seawater (Vector-Borne National Center, 2008).

Epidemiological data reveals a correlation between preexisting liver disease and the occurrence of primary septicemia caused by *Vibrio parahaemolyticus* with an observed fatality rate of forty-four percent for cases with septicemia (Hlady and Klontz, 1996). Patients may incur wound infections before or during exposure to seawater or seafood drippings when *Vibrio parahaemolyticus* is present (Qadri, et al., 2005). Ear infections, eye infections, and peritoneal infections have also been associated with *Vibrio parahaemolyticus* in recent studies (Hornstrup and Gahrn-Hansen, 1993; also Daniels, 2000).

Person-to-person infectious transmission may occur through direct physical contact, or indirectly through the secondary contamination of household food and water (Qadri, et al., 2005). Hornstrup and Gahrn-Hansen studied the frequency of mother-to-infant transmission of enteric pathogens and documented the isolation of *Vibrio parahaemolyticus* (Duangmani, et al., 1985).

## The Global Human Health Perspective

*Vibrio parahaemolyticus* has surfaced around the world as a major threat to public health. Bacterial clones of *Vibrio parahaemolyticus* with the potential for causing pandemic infections have emerged in Asia (Matsumoto, et al, 2000). Internationally and domestically, *Vibrio parahaemolyticus* is one of the most significant vectors of bacterial foodborne illness.

Table 1 Cross-comparison of Three Prevalent Pathogenic *Vibrio* Species[1]

| Genus | Species | Hosts of Disease | | Routes of Human Infection | | | Estimated Infection Doses | Human Syndrome | References |
|---|---|---|---|---|---|---|---|---|---|
| | | Marine Animals | Human | Aerosol | Zoonoses | Seafood | | | |
| *Vibrio* | *V. cholerae O1* | X | X | X | X | | $10^6 – 10^{10}$ cells | GI, sepsis | Pavia et al., 1989 |
| *Vibrio* | *V. vulnificus* | X | X | X | X | X | $10^3$ - $10^5$ CFU/g | GI, wound/ear infection, sepsis | Howard & Bennett, 1993 |
| *Vibrio* | *V. parah-aemolyticus* | X | X | | X | X | $10^6$ CFU/g | GI, wound/ear infection, sepsis | CDC, 1999, 2000 |

[1] Thompson, Marceline, Polz, 2005, 34-35.

Table 2 Cross-comparison of Immunological and Molecular Methods for Detection of Environmental *Vibrio* Species Samples[2]

| Bacteria | Sample | Target | Method | Selectivity | Reference |
|---|---|---|---|---|---|
| *Vibrio cholerae* | Raw oysters | Hemolysin (*hlyA*) | Real-Time PCR | $10^2$ CFU/100ml | Lyon, 2002 |
| | Waste Waters, estuarine waters--Environmental isolates | *ctxA*, *hlyA*, *ompU*, *stn/sto*, *tcpA*, *tcpI*, *toxR*, and *zot* genes | Multiplex PCR | No Data | Rivera et al., 2001 |
| | Water/food outbreaks—clinical isolates | Cholera enterotoxin, gene (*ctx*) | ELISA (US-FDA established protocols) | No Data | Jackson, et al., 2000 |
| | Sewage polluted seawater | *ctx* gene | PCR | 10-100 CFU/reaction | Kong et al., 2003 |
| | Seawater | ctxA, 16s-23s rRNA | PCR | No Data | Lipp et al., 2003 |
| | Estuarine water | O1 or O139 surface antigens | Agglutination following enrichment | $2x10^3$ CFU/ml No Data | Hassan et al., 1995, Louis et al., 2003 |
| | Seawater | 16s-23s rRNA | Colony hybridization following enrichment | No Data, but <100 CFU/100ml was detected | Jiang and Fu, 2001 |
| *Vibrio vulnificus* | Seawater, sediment, fish | Cytolysin gene | Hybridization following culture enrichment | 2 CFU/100ml | Hoi et al., 1998 |
| | Oysters-isolated strains | Genome | PFGE | No Data | Jackson et al., 1997; Tamplin et al., 1996 |
| | Eels | LPS | ELISA | $10^4$-$10^5$ cells/reaction | Biosca et al., 1997 |
| | Water | Hemolysin (*vvhA*) gene | Colony hybridization | $2x10^3$/100ml | Lee et al., 2001 |
| *Vibrio parah-aemolyticus* | Raw oysters | Thermostable direct hemolysin (*tdh*) gene | Colony hybridization--culture enrichment | 10 CFU/g D | DePaola et al., 2000; Nordstrom & DePaola, 2003 |
| | Raw oysters | *tdh* gene | Real-time PCR | 1 CFU/reaction | Blackstone et al., 2003 |
| | Sewage polluted seawater | 16s-23s rRNA | PCR | 10-100 CFU/reaction | Kong et al., 2003 |
| | Seawater | *toxR*, *tdh*, *trh* genes | PCR | 3 cells/100ml | Alam et al., 2003 |
| | Seafood | *tdh* gene | PCR with enrichment | 0.3 cells/g | Hara-Kudo et al., 2003 |

[2] Thompson, Marceline, Polz, 2005, 46-47.

Gastroenteritis that is associated with *Vibrio parahaemolyticus* has been reported throughout North America, Central America, Europe, Asia, and Africa. Numerous studies have shown that the *Vibrio parahaemolyticus* organism is pathogenic for human populations residing in a variety of geographic locations. In Asia, *Vibrio parahaemolyticus* is an established enteric pathogen in Japan, where consumption of uncooked seafood is common. As a result, scientists have concluded that *Vibrio parahaemolyticus* is responsible for 50-70% of the cases of Japanese bacterial food poisoning (Miwatani and Takeda, 1976; Obata, et al., 2001).

The isolation of *Vibrio parahaemolyticus* in patients suffering from diarrhea has been reported in Thailand, India, Bangladesh, Laos, Vietnam, Tanzania, Hong Kong, Korea, Russia, China, Taiwan, Indonesia, Philippines, and Kuwait. In Europe, *Vibrio parahaemolyticus* has been discovered in the Baltic Sea, the North Sea, the Mediterranean Sea and the Black Sea (Miwatani and Tekeda, also Molero, et al, 1989). However, the greatest local implication of Vibrio infections in the United States has been directly correlated to five U.S. coastal states (Figure 1). *Vibrio* infections per resident is by far highest in the state of Hawai`i. This can be due to the fact that Hawai`i is the only state listed with year-round tropical climate, or due to the influx of over seven million visitors to Hawai`i's beaches and coastal systems. However, percent deaths affiliated with *Vibrio* infections still remains higher in areas where consumption of unsafe shellfish is prevalent.

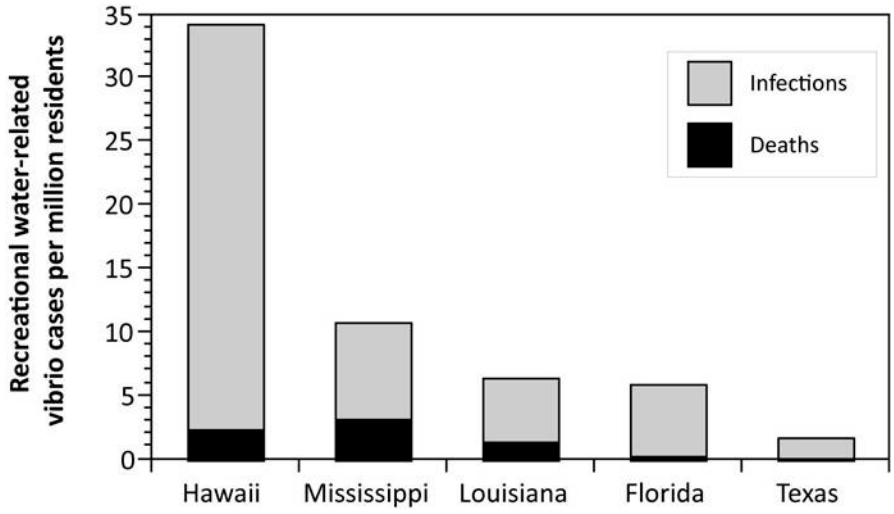


Fig. 1 Vibrio Infections of Five U.S. States per Resident Population[3]

**Introduction to Study Site and Local Environment**

Located in Honolulu, Hawai'i on the island of O'ahu (Figure 2), the Ala Wai Canal was built in 1928 as an artificial waterway for the purpose of draining rice paddies, fish and duck ponds, and swamps. At present, the Ala Wai Canal diverts stream flow from low-lying land area adjacent to one of Honolulu's ocean harbors. The Ala Wai Canal was originally proposed in 1906 by Lucius Pinkham, who was president of the Hawai'i Board of Health. Pinkham envisioned this low-lying land as a potential site for resort and recreational facilities. He suggested that the Ala Wai Canal would consolidate the discharge flow and sediments of the Makiki, Manoa, and Palolo streams. A man-made canal would divert these from streams from flooding the area which would become Waikiki. Construction on the canal began in 1922 and was completed four years later in 1926. In the Native Hawaiian language, "Ala Wai" means *"Path to the Sea."*

[3] Yoder, et al., 2008; Dziban, et al., 2006; and compiled by Steward, 2008

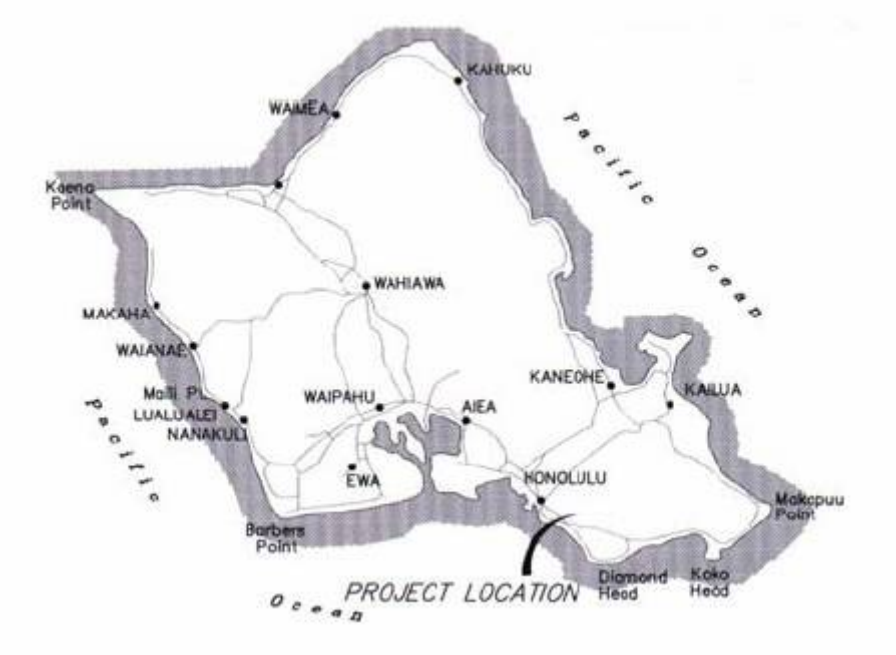


Fig. 2 Map of O`ahu, Hawai`i. (DLNR, 2004)

*Ala Wai Watershed*

The Ala Wai Watershed (Figure 3) is comprised of an area that is approximately 12,033 acres. It includes the sub-watersheds of Makiki, Mānoa, and Pālolo. The watershed extends from the top of the Ko'olau Mountains to the near-shore waters of Waikiki and Māmala Bay (*U.S. Army Corps of Engineers*, 2005). The canal runs parallel with the development of Waikiki and concludes at the Waikiki Boat Harbor. Three bridges span the Ala Wai Canal. These transportation corridors are at the McCully Street, the Kalākaua Avenue, and the Ala Moana Boulevard crossings.

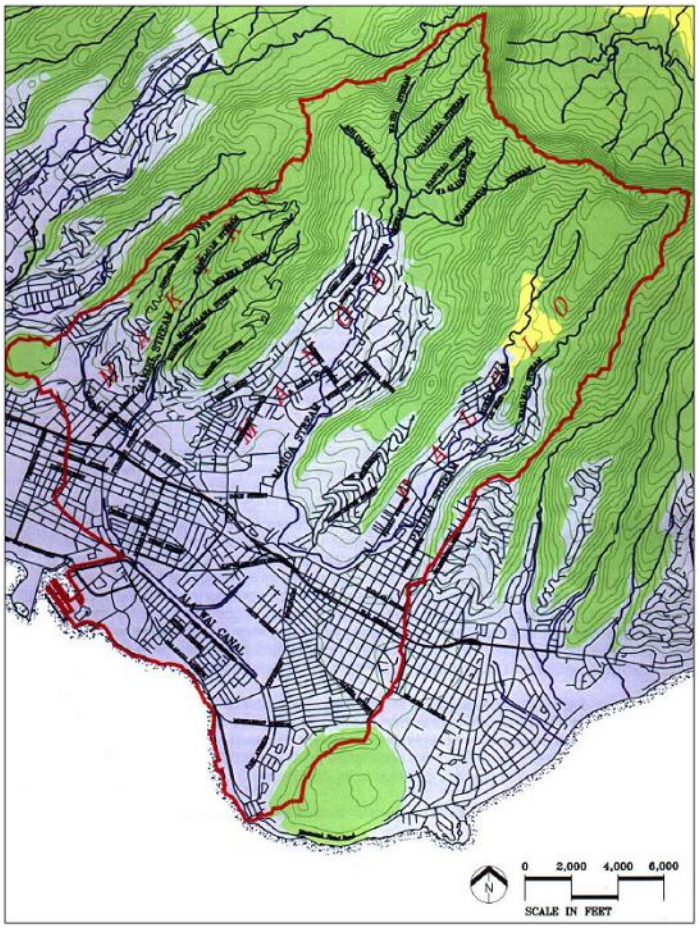


Fig. 3 Topographic Map of Ala Wai Watershed Area O`ahu, Hawai`i. (DLNR, 2004)

*Recreational Water Use*

The Ala Wai Canal is used recreationally by paddlers, canoe teams, and other navigators of non-motorized boating vessels. Although fishing is prohibited throughout the canal, throughout our study we observed many local fishermen using the canal to collect bait fish and other small fry. The Ala Wai Boating harbor is the point of discharge for one the largest recreational boating facilities of the State of Hawai'i, with a berthing capacity of 699 vessels. Located within this harbor are the Hawaiian Yacht Club, the Waikiki Yacht Club, and the Royal Hawaiian Ocean Racing Club.

*Climate*

Honolulu, Hawai'i is located at 21º 18' N, 157º 51' W. Hawai`i's monthly mean air temperatures, like most global land areas, vary seasonally. At sea level, average daytime air temperature varies from about 25.5 degrees Celsius in August, to 22 degrees in February. Typical coastal sea water temperatures for Honolulu, Hawai'i range from 24.5°C to 26.6°C (Figure 4).

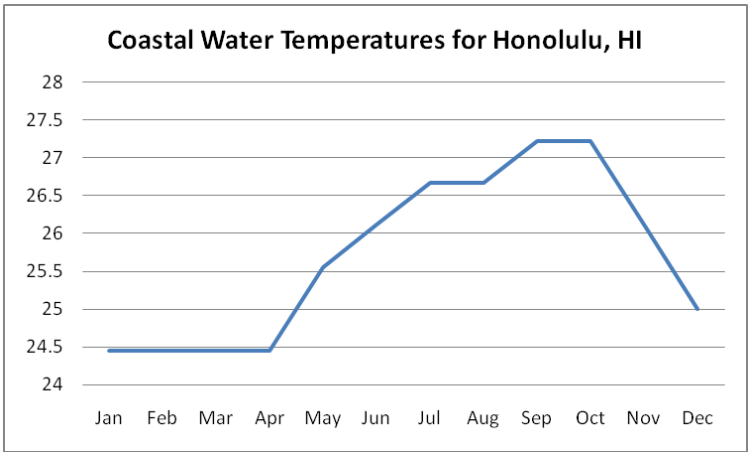


Fig. 4 Average Monthly Coastal Water Temperatures (°C), Honolulu, HI[4]

## Vibrio Dynamics of Marine Environments

*Vibrio parahaemolyticus* not only negatively affects human health, but *Vibrio* bacterium also influences the nutrient cycling and remineralization of organic nutrients in the environment. Every *Vibrio* species abundance is ecologically dependent on three main factors: water temperature, salinity, and the concentration of planktonic organisms. Most species of *Vibrio* are characterized by increased growth at elevated temperatures.

[4] Coastal Water Temperature Table, National Oceanographic Data Center.

As a result, higher rates of isolation may be observed in the marine environment during warm months (Lipp, et al., 2002) and in the presence of planktonic copepods (Huq, et al., 1990). A study detailing the seasonal distribution of *Vibrio parahaemolyticus* concluded that an increase in isolation directly paralleled an increase in water temperature (Sarkar, et al., 1985).

The *Vibrio parahaemolyticus* bacterium exists either as a free living organism or attached dependent to submerged, inert, and animate surfaces such as particulate matter, zooplankton, fish, and shellfish (Qadri, et al., 2005). Like many *Vibrios, V. parahaemolyticus* may be found as a planktonic, free-swimming state and a sessile state on a host surface.

In 2003, Stewart and McCarter described various cell movements allowing *Vibrio parahaemolyticus* to swim freely in marine surroundings.

> The swimmer cell, with a single polar flagellum, is adapted to life in liquid environments. The polar flagellum, powered by the sodium motive force, is constitutively expressed and propels the bacterium at various speeds, allowing it to find and closely approach a surface. The swarmer cell, propelled by many proton-powered lateral flagella, can move through highly viscous environments, colonize surfaces, and form multicellular communities.[5]

Marine copepod exoskeleton has been shown to support large populations of *Vibrio*, including the pathogenic species of *Vibrio cholerae, Vibrio vulnificus, Vibrio alginolyticus and Vibrio parahaemolyticus* (Carli, et al., 1980; also Colwell and Huq, 1999; Hansen and Bech, 1996; Huq, et al, 1983; Tamplin, et al., 1990; Kaneko and Colwell, 1975). Recent evidence suggests a mutualistic symbiosis between species of copepods and *Vibrios* (Lipp, et al., 2002). Since *Vibrio parahaemolyticus* produces an

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[5] Stewart and McCarter, 2003: 232.

active chitinase, which breaks down the chitin produced by the copepods, the ecological significance of the organism is presumed to involve the recycling of chitinous material, as well as other organic nutrients derived from zooplankton (Kaneko et al., 1974).

In a comparable study (Huq, et al, 1983), Huq found that *Vibrio parahaemolyticus* can bind uniformly to surfaces of copepod exoskeleton. Colwell (1981, 1986) highlights the important compensational symbiotic relationship between the *Vibrios* and copepods into four distinct benefits to *Vibrios*. First, *Vibrio* bacteria bound to the exoskeleton are able to metabolize the chitin more efficiently than free-living forms could do normally. Second, while the bacterium is attached to copepods and, in particular, to the eggs that are being dispersed in the water, provided a mechanism for extended geographic distribution. Third, plankton organisms are the most plausible reservoir of altered forms of pathogens, from which fully virulent strains can arise. And finally, bacteria that grow on the surface of a copepods are protected against environmental stress and thrive on excreted products of digestion, including organic material and $NH_4^+$ (Cooksey and Wigglesworth-Cooksey, 1995).

The interaction of various *Vibrio* species with copepods was found to be influenced by certain environmental parameters (Kaneko and Colwell, 1975). For example, as concentrations of salinity increased, the total number and rate of *Vibrio parahaemolyticus* which attached to the copepods decreased.

Species of *Vibrio* can be entrapped by filter feeding invertebrates. These marine animals sieve suspended food particles from their aquatic environment (Pruzzo, et al. 2005). This filter feeding process may cause internal concentrations of potentially pathogenic bacteria to increase. This can establish a commensal relationship with the

host, or the *Vibrio* can proliferate and invade the soft tissues on the invertebrate causing death. The prevalence of bacteria in filter feeders, as well as in other shellfish, largely depends on their sensitivity to the hemolymph bactericidal activity (Cuthbertson, et al., 2002; also Destoumieux, et al., 2000; Harris-Young, et al., 1995; Olafsen, 1995; Prieur, et al., 2002).

A recently reported optimum salinity for *Vibrio parahaemolyticus* growth was estimated to be 23 parts per thousand (ppt) (Anonymous, 2005). Despite its halophilic nature, *Vibrio parahaemolyticus* has been isolated from saline-free waters, and its occurrence in freshwater systems has been construed as a accidental incidence that is probably related to tidal drift of organisms to the upper reaches of rivers (Sarkar, et al., 1985). *Vibrio parahaemolyticus* grows well in media supplemented with 20-20‰ sodium chloride and preferentially inhabits brackish aquatic environments (Pruzzo, et al., 2005). Yet, under specific nutrient conditions, sodium ion requirements are not mandatory and *Vibrio parahaemolyticus* can survive well in areas where salt concentration is lower than physiological concentrations (Sarkar, et al., 1985).

Along with *Vibrio vulnificus, Vibrio parahaemolyticus* has been shown to be isolated in water of a temperature that is higher than 10°C (Oliver, et al., 1983). However, other studies have shown that *Vibrio cholerae* and *Vibrio parahaemolyticus* survive at lower temperatures in the presence of chitin. This suggests that chitin may possess a cryoprotective capacity allowing *Vibrio* to be maintained during winter months (Amako, et al., 1987; also Karunasagarm, et al., 1986). Overall, *Vibrio parahaemolyticus* abundance varies with temperature, but recent data suggests that highest densities of cells occur in waters ranging from 20°C to 30°C (Tantillo et al., 2004).

## Isolating and Identifying of *Vibrio parahaemolyticus*

### *Conventional Cultivation Methods*

Numerous molecular-based approaches are being employed to identify the presence *Vibrio parahaemolyticus.* These relatively new systems have been developed to efficiently, reliably and accurately detect microbial pathogens in the environment. Even in the remotest parts of the world, researchers and practitioners are utilizing these molecular means (Pruzzo, et al., 2005). As well, conventional methods, such as the ones used in this study, are used to detect and classify *Vibrio parahaemolyticus* isolates from clinical and environmental samples.

Two types of *Vibrio*-selective media are frequently utilized: Thiosulphate-Citrate-Bile-Salts-Sucrose agar (TCBS) and CHROMagar™ *Vibrio* (CV) agar. After colonization and isolation of individual isolates, species identification is established using PCR assays. The bacterial colonies are screened for the occurrence of species-specific gene sequences. The analysis of 16S rRNA sequences is acknowledged as the "gold standard" of microbial niche scanning (Amann and Schleifer, 1994).

TCBS allows for the selective isolation of Cholera *vibrios* and *Vibrio parahaemolyticus* from a variety of clinical and non-clinical specimens (Downes and Ito, 2001; also Clesceri, et al., 1998). The high concentrations of thiosulfate and citrate and the strong alkalinity of this medium largely inhibit the growth of *enterobacteriaceae* (Kobayashi, Enomoto, Sakazaki, Kuwahara, 1963). Ox bile and cholate suppress the development of *enterococci* and coliform bacteria by preventing the metabolizing of sucrose.

The downside of using TCBS is that this method of bacterial detection requires a lengthy time for colony formation and screening for proper colony identification. In contrast, the medium of CV facilitates the identification of *Vibrio parahaemolyticus, Vibrio vulnificus* and *Vibrio cholerae* from other *Vibrio* species. The bacterium are isolated by their colony colors with a higher sensitivity than conventional methods (Rambach, 2008). The CV medium is selective against most major *enterobacteriaceae* and Gram positive bacteria.

## *Molecular Assays*

Molecular diagnostic tests have been developed over the past few years for both the clinical and environmental monitoring of *Vibrio parahaemolyticus* pathogen types. However, conventional methods of pathogen detection such as the most-probable-number technique in association with a biochemical test, or the procedure of colony blot hybridization with gene-specific probes, are both time and labor consuming when analyzing a large number of samples (Kaysner, et al., 2001). These microbiological challenges are further complicated by the phenotypic variations within species, the scores of newly described pathogenic species, and the restricted array of phenotypic tests available to distinguish established and potential bacterial pathogens.

### 16S rRNA

The sequence of the 16S rRNA gene has been widely used as a molecular clock to estimate relationships among bacteria (phylogeny). Additionally, 16S rRNA has become an important means of identifying unknown bacterium at the genus or species level (Sacchi et al., 2002). The 1542 nucleotide-long 16S gene is a relatively small component

of the prokaryotic ribosomal subunit (30S). The 16S primers sequence, listed below in Table 3, bracket a highly variable nucleotide sequence region which can provide species-specific signature sequences effective for bacterial identification.

## Thermolabile Hemolysin

The thermolabile hemolysin (*tlh*) gene is a species-specific marker that may be used as a means to detect pathogenic strains of *Vibrio parahaemolyticus* (Parveen et al., 2008). Detection and quantification of the *tlh* gene represents only the presence of the specific gene and the possibility of pathogenic virulence.

## Thermostable Direct Hemolysin

The *tdh* gene is a proven virulence factor (Nishibuchi et al., 1992), which occurs in over ninety percent of the clinical strains isolated in the United States and the world (Okuda et al., 1997). The *tdh* gene encoding the thermostable direct hemolysin (TDH) is regarded as an important virulence gene. Most clinical strains carry this *tdh* gene, yet it is contained in only a small proportion of environmental strains (Nishibuchi and Kaper, 1995, M. Nishibuchi and J.B. Kaper).

An earlier method known as the Kanagawa test was first used in early studies to help categorize strains producing the *tdh* target gene. However, this test been substituted for more efficient diagnostic methods such as PCR and DNA probe assays (Bej et al., 1999 and DePaola et al., 1994). These approaches and other genotypic assays have been successfully applied to both environmental and seafood studies.

## Significance of the Research

Three core questions remain unanswered about *Vibrio parahaemolyticus* and the Ala Wai estuarine system. First, is *V. parahaemolyticus* present in the waters in and around the Ala Wai canal? Second, if present, what environmental factors influence *V. parahaemolyticus* abundance? And lastly, do the strains of this bacterium growing in the canal carry a known virulence-associated gene? Answers to these questions will be helpful in evaluating whether *V. parahaemolyticus* presents a risk of infection for individuals that interact with the waters in and around the Ala Wai canal. Data on the relationship between the abundance of *V. parahaemolyticus* and environmental conditions may also prove useful for future models *Vibrio* spp. dynamics in this urban estuarine environment and how risks of infection vary over space and time.

# CHAPTER 2

# MATERIALS AND METHODS

## Overview of Collection Design

### *Sampling Strategy*

Samples were collected at fifteen sites along the shores of the Ala Wai Canal, the Ala Wai Boat Harbor, and the streams that feed into the canal on nested time scales ranging from months to hours. Sites were distributed along the Ala Wai Canal in order to capture effects of some of the major freshwater inputs to the canal such as streams, and storm drains.

The strategy of this project's sampling design was to accommodate the possibility that variations of time could affect the content of the samples being collected and analyzed. Therefore, a time series of monthly collections was taken in 2008 of water samples from each station. Additionally, a nested hierarchical time series of water sample collection was implemented. In this hierarchical scheme, water was collected at varying times of weekly, daily, and three-hour frequencies. The three hour collection cycle was limited to a twenty-four period of time in July. Samples were collected during the morning to early afternoon hours. For logistical reasons, daily samplings were limited to four stations and the twenty-four hour sampling was limited to three stations.

The nested hierarchical time series was limited to only a select few stations during the intensive month of July. Since sampling all fifteen stations was not possible for weekly, daily and hourly sampling; preselected groups of sampling stations were designated for collection. The sample collection was concluded in November 2008.

The weekly collection sites were limited to One and Five through Fifteen. Sites Two through Four were excluded from the weekly study because Stations Two and Three were at the same location as Site One, however at a different depth. Site Four was also excluded because it was deemed unnecessary to collect sediment samples on the week-to-week scale.

The daily sample series ran from July 10 through July 17, 2008. During these seven days samples were collected from Stations Five, Nine, Twelve and Fourteen. These four stations were preselected due to each sites specific location to key water inputs and samplers accessibility. Station Five was located at the far end of the canal, near the out-rigger launching ramps. Station Nine was located behind Io`lani School on a small dock in Mānoa stream. Station Twelve was located ten to twelve yards from where the Makiki stream flows into the Ala Wai Canal. Finally, Station Fourteen was located on the west side of the canal around twenty yards upstream from the Ala Moana Boulevard bridge.

Hourly sampling began on July 15th at 6:00 a.m. and was completed on July 16th at 6:00 a.m. Similar to the daily sampling, the hourly sampling involved a limited preselected group of collection sites: Stations Five, Nine, and Fourteen. Station Twelve was omitted because there was a lack of reliability for the researcher to access the site during the twenty-hour period. Samples were collected during the morning to early afternoon hours.

Table 3 Nested Hierarchal Time Series

| | | |
|---|---|---|
| ***Monthly*** | Stations 1-15 | February through November 2008 |

| | | |
|---|---|---|
| ***Weekly*** | Stations 1,5-15 | June 26/08 through July 17/08 |
| ***Daily*** | Stations 5,9,12,14 | July 10/08 through July 17/08 |
| ***Hourly*** | Stations 5, 9, 14 | July 14/08 at 0600 through July 15/08 at 0600 (3-hour intervals) |

In consideration of the significant variability of the tidal flux, each sampling day was correlated to the most consistent tide from other sampling days. However, in some cases it was unnecessary or improbable to base variability on consistent tidal markings.

*Location of Stations*

A satellite-view representation of the fifteen sampling locations along the Ala Wai Canal is provided in Figure 5.


Fig. 5 Sampling Station Locations (1-15) Along the Ala Wai Canal, Honolulu, HI[6]

## Sample Collection

### *Water*

The fifteen collection stations were located within five feet of the shore line. Stations One through Nine and Station Eleven were accessible without the use of an extendable pole; while Station Ten and Stations Twelve through Fifteen required the use of the pole to reach the collection site in the water.

At most of the collection sites, a plastic bottle was completely submerged to a depth of between one and two feet of water. However, at collection Stations One through

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[6] Tele Atlas North America, Inc, 2008.

Four and Station Seven, a full-submersion of the bottle was not possible due to low water levels because of tidal activity, rocky surfaces, or other restrictions. In these cases, the bottle was submerged closer to the level of the water's surface.

Before each collection was taken, the bottles were thoroughly flushed with water to ensure the absence of any prior bottle contamination. During the flushing of each of the bottles, the excess water was not returned to the collecting system so as to prevent the mixing of discharge water with collection water and to minimize sediment disturbance.

### *Sediment*

Sediments were collected at one site only (Station four). Station Four was geographically isolated from any disturbing stream flow, anthropogenic activity, or other turbid inputs that could potentially skew an analysis of the sediment. Sediments from Station Four were collected where the water depth was approximately five to eight inches by scooping with a sterile polypropylene screw-cap tube (15-ml capacity).

## Instruments and Tools

While in field, it was necessary to utilize several different collection and processing tools. A basic pre-calibrated standard glass alcohol (0°C to 80°C) thermometer a digital pocket refractometer (PAL-1 by Atago U.S.A., Inc.), falcon tubes (15 ml) and pipettes were employed. A retractable sampling pole (Swing Sampler, Nasco) was employed to reach areas where the water was beyond arm's reach.

One-liter brown non-transparent bottles and 500ml transparent bottles were used at each of the stations. Every bottle that was used to collect and analyze the water

samples was pre-labeled, autoclaved and/or acid washed (10% HCl solution for twenty-four to forty-eight hours).

The refractometer was calibrated with NanoPure water. Once calibrated, a 100 microliter sample of surface water from each station was collected and placed on a refractometer for measurement. Salinities were routinely measured from the sample bottles once they had been returned to the lab. At Station One, surface salinity was also routinely measured in the field at two depths by directly pipetting from the upper centimeter and from ca. 5-10 cm depth (near the bottom) to determine whether the water was stratified.

At every site, care was taken to minimize disturbance of the sediments during the water sampling. Each bottle was capped and placed in a pre-chilled ice cooler with lid. The immediate cooling of the water samples was necessary to slow down and/or prevent further chemical and biological activity from occurring. With each sample a thorough recording of the site location, water temperature, and time of day was documented.

Station Four stands alone as the only site at which sediment samples were collected on three different occasions. The sediments were placed in a 15 ml sterile falcon tube and appropriately labeled. In such cases, it was necessary to determine and record the sediment temperature, as well as the water-surface temperature.

## Management of Samples

After each sampling, the water and sediment collections were immediately transported to the laboratory of the University of Hawai'i for analysis and processing. Samples were transported to the laboratory at 4-10 degrees Celsius, within sterilized containers, and were processed within the six hour standard prescribed by Donovan and

Van Netten for the collection timeline of the *Vibrio* species (1995). All samples were processed to a point where they could be stored within three to four hours.

*Sterivex Filtration*

Water samples were filtered through 0.22 µm pore-size filters (Sterivex, Millipore) in order to collect bacteria for subsequent cultivation-independent molecular analyses. Filtrates were saved for the future isolation of bacteriophages infecting pathogenic vibrios.

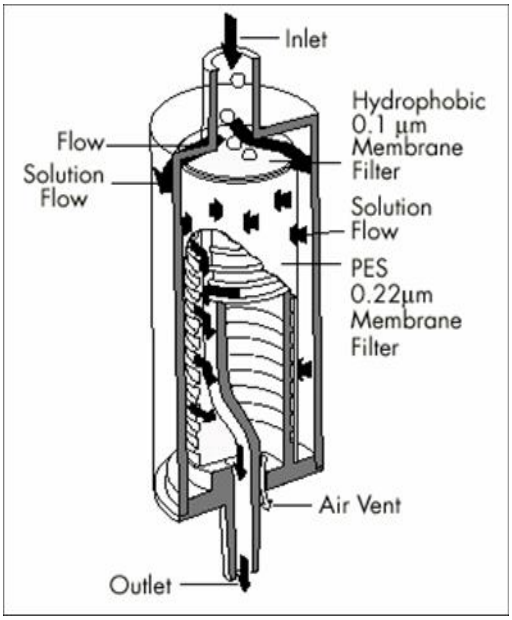


Fig. 6 Sterivex-GP unit: Cross-Sectional View (*Millipore Corporation*, 2008).

An eight-lane peristaltic pump with containing tubes was connected with a 0.22 µm sleeve to inlet of the Millipore Sterivex™-GP filtration unit. Semi-transparent 500ml samples bottles were used to collect filtrated water from the outlet of the Sterivex-GP

Unit. Each containing tube was labeled at both ends. Prior to their use, the containing tubes were soaked in a 2% Contrad liquid detergent bath solution with Nanopure water. The peristaltic pump was then turned on at a conservative rate to prevent tube wear and/or filter hemorrhaging. During the filtration of the water samples, a sterile 50ml Falcon tube was placed beneath the Sterivex filter and used to collect any excess water. After each Falcon tube was sufficiently filled, it was labeled and placed in a 4°Celcius refrigerator. This filtered water would then be available for further viral analysis.

Once 500ml of sample water had been filtered through the Sterivex unit, the filter was removed and the capsule of the unit was examined for any remaining liquid. If any water remained, a 10-20ml sterile syringe was connected to the filter unit and the remaining solution was removed. Each end of the Sterivex filter was then covered with a self-sealing Parafilm® M Cover Film, and labeled according to sample. These bottles were stored in a -80°Celcius freezer for future analysis.

### *Collection Management and Storage for Future Analysis*

#### Total Organic Carbon

Total Organic Carbon (TOC) describes the sum of all the organic carbon-containing constituents (whether living or dead) found in a sample. Total Organic Carbon (TOC) is often used as a non-specific indicator of water quality (Mauri, 2007). The samples from each site were placed in a 30ml, pre-acid washed, wide-mouth Nalgene HDPE container and frozen at -25°C for subsequent analysis by high-temperature combustion.

Particular Organic Carbon

Sample water (25-200 ml) from the non-transparent one-liter bottles was filtered onto pre-combusted glass fiber filters (GF/F, Whatman). The amount of water that was processed was determined by the particulate load in each sample. A log was maintained of how much water was processed from each collection station. After filtration was complete, filters were removed with sterile forceps and folded in half (sample side facing inward) inside a sheet of aluminum foil. The edges of the aluminum sheet were folded to contain the filter, then the foil packets were labeled and stored in a sterile plastic bags (Whirlpak, Nasco) and placed in a -80°C freezer.

Chlorophyll *a*

Samples for analysis of extracted chlorophyll a were processed and stored identically to those for POC, except that the filters were not pre-combusted.

Untreated water samples were collected and placed in a acid-washed 30ml, semi-transparent Nalgene screw-cap bottle. Each bottle was immediately placed in -20° Celsius freezer. The recommended shelf-life for these containers was deemed to be no more than three to four months.

Dissolved Iron

Sample water from each station (20 ml) was filtered through a 0.2 µm polysulfone membrane, syringe-tip filter (Pall, Acrodisc) using sterile, plastic syringes. The filtrate was collected in a 25ml acid-washed semi-transparent screw cap bottle, containing 200µl of Optima-Grade Hydrochloric Acid.. These bottles containing the water samples were then stored in a refrigerator at 4°Celcius.

## Isolation and Analysis of *Vibrios*

Water samples were filtered through a pre-sterilized Pall Corp.® QH-6 Grid filter (0.45µm, 47mm) in a biological safety cabinet and the filters were placed face up on to two types of *Vibrio*-selective media: Thiosulfate Citrate Bile Sucrose Agar (TCBS) and CV *Vibrio*. Plates were incubated at 37°C for eighteen to thirty-four hours, After incubation, the number and color of the colonies on each plate was recorded.

Individual mauve (on (CV) or green (on TCBS) colonies were collected with sterile inoculating loops and transferred to new plates. Colonies from one media were transferred to the other media to document the color of each isolate on both media. Colonies that were mauve on CV and green on TCBS were considered to be presumptive *V. parahaemolyticus*.

From each of the fifteen stations, four separate colonies were collected and streaked on four different duplicate plates, for a total of sixty plates. After a sufficient incubation period, the newly streaked plates were counted for colony-forming units (CFU) and then restreaked for a third and final time. If at any point the colony-forming units either changed to an inappropriate color or did not grow, then plates were deemed unusable (i.e. contamination, over-incubation, or plates that were too numerous to properly isolate).

After restreaking three times, all isolates that consistently formed green colonies on TCBS and mauve colonies on CV were transferred using sterile inoculating loops to slants. Slants consisted of four to five milliliters of TCBS solidified at an angle in the bottom of a 10-ml capacity sterile culture tube. Each tube was labeled according to the isolate and placed into an incubator. After sufficient growth, each tube was filled with five to eight milliliters of autoclaved mineral oil and be stored at room temperature.

A portion of a colony of each of the final isolates was also individually collected with a sterile loop and transferred into 96-Well Plate filled with a 100µl 1x-concentrate TE buffer solution and stored in at -20 °C.

*Vibrio*-selective Media: Thiosulfate Citrate Bile Sucrose Agar

The presence of microorganisms can be detected according to the specific coloration of colonies present on Thiosulfate Citrate Bile Sucrose (TCBS) media. The table that follows describes the color identification process:

Table 4 Thiosulfate Citrate Bile Sucrose Agar Color Identification Used[7]

| ***Color of Colonies*** | ***Microorganisms*** |
|---|---|
| Yellow | *Vibrio cholerae*<br>*Vibrio alginolyticus* |
| Green | *Vibrio parahaemolyticus*<br>*Vibrio vulnificus* |

This coloration enabled the researcher to isolate and selectively cultivate *Vibrio cholerae, Vibrio parahaemolyticus,* and other *vibrios*. According to their utilization of sucrose in the agar medium, the genus of the *vibrio* colonies was distinguished and separated into two major groupings. Yellow colonies were indicative of the possible presence of *Vibrio cholerae, Vibrio alginolyticus,* or *Vibrio fluvialis*. Green colonies (sucrose negative) represented the presence of *Vibrio parahaemolyticus, Vibrio vulnificus,* and *Vibrio mimicus* (Percival, et al., 2004). Within conventional isolation media, Thiosulfate Citrate Bile Sucrose (TCBS) Agar differentiates the two species of *Vibrio parahaemolyticus* (sucrose-negative) and *Vibrio alginolyticus* (sucrose-positive) ninety-nine percent of the time (Oliver and Kaper, 2000).

*Vibrio*-selective Media: CHROMagar *Vibrio*

Similarly, after the incubation of each plate, isolate colony growth counts were determined in CV media. The incubation period was for eighteen to thirty-four hours at 37°Celcius. Table 6 describes how the color classification of the CV media was used to identify microorganism colonies.

[7] *Merck KGaA.*

Table 5 CHROMagar *Vibrio* Color Identification Used[8]

| ***Color of Colonies*** | ***Microorganisms*** |
|---|---|
| Mauve | *Vibrio parahaemolyticus* |
| Turquoise-Blue | *Vibrio cholerae*<br>*Vibrio vulnificus* |
| Colorless | *Vibrio alginolyticus* |
| Inhibited or colorless | Other bacterial colonies |

## PCR Assays

### *Sample Preparation*

Polymerase Chain Reaction (PCR) is a molecular technique that can be used to determine the presence of *Vibrio parahaemolyticus* through several subsequent assays. The technique amplifies a specific piece of DNA by *in vitro* enzymatic replication. PCR involves three steps: DNA extraction, amplification and Gel electrophoresis.

To extract the DNA, a 96-Well Plate was filled with a 100µl 1x-concentrate TE buffer solution and placed in a Thermal Cycler PCR machine and set to run at programmed temperature cycle for fourteen minutes. This initial step separated the DNA from loose cell material.

The 96-well plate was then transferred to a refrigerated (5°Celsius) centrifuge and spun for five minutes at 5000 rpm. From each well, 40µl from the top-most layer was transferred to a second 96-Well Plate. The second plate was labeled and stored in -70°Celsius freezer for further analysis.

[8] Rambach.

*PCR Amplification*

PCR amplification were carried out according to the instructions designated by the Taq manufacturer (Roche).

Table 6 DNA Primers Used for PCR and DNA Array Hybridization

| Pathogen | Target Gene | Primer and Probe Sequence | Oligonucleotide | %GC content | Tm (°C)[b] | Amplicon size[a] (kbp) | References |
|---|---|---|---|---|---|---|---|
| *V. parahaemolyticus* | 16s | F-16s: 5' –AGAGTTTGATCCTGGCTCAG-3' | 20 | 50 | 50.0 | 1.492 | |
| | | R-16s: 5' –ACGGCTACCTTGTTACGACTT-3' | 21 | 47.6 | 72.0 | | |
| | [b] *tlh* | F-TLH: 5'-AAAGCGGATTATGCAGAAGCACTG-3' | 24 | 45.8 | 62.8 | 0.45 | (Nishibuchi and Kaper, 1985) |
| | | R-TLH: 5'B-GCTACTTTCTAGCATTTTCTCTGC-3' | 24 | 41.6 | 61.1 | | |
| | [c] *tdh* | F-TDH: 5'-GTAAAGGTCTCTGACTTTTGGAC-3' | 23 | 43.5 | 57.7 | 0.269 | (Honda and Iida, 1993) |
| | | R-TDH: 5'B-TGGAATAGAACCTTCATCTTCACC-3' | 24 | 41.7 | 61.1 | | |

[a]*kbp* – kilobase pairs
[b]*tdh* – thermostable direct hemolysin
[c]*tlh* – thermolabile hemolysin

For each isolate, the following materials were added in subsequent order:

i. 42.15µl Nuclease Free Water

ii. 5µl 10x Buffer

iii. 1µl 10mM DNTP

iv. 0.3µl Forward primer

v. 0.3µl Reverse primer

vi. 1µl template

The specific primers used varied according to the assay (Table 6). Reaction mixtures were transferred into the wells of a PCR 8-well strip and placed in a thermal

cycler. The cycling conditions varied slightly for each of the three reactions and are summarized in Table 7.

Table 3 PCR Conditions for 16S rRNA, *tlh* and *tdh* PCR amplifications

| Gene Amplification Type | Primers (bp): | PCR Temperature Conditions | Time | No. of Cycles |
|---|---|---|---|---|
| 16S | 1492 | 95°C | 5 min | 1 |
| | | 95°C<br>50°C<br>72°C | 1 min<br>1.5 min<br>2 min | 30 |
| | | 4°C | ∞ | 1 |
| | 450 | 95°C | 5 min | 1 |
| | | 95°C<br>58°C<br>72°C | 1 min<br>1 min<br>1.5 min | 40 |
| | | 72°C | 7 min | 1 |
| | | 4°C | ∞ | 1 |
| *tdh* | 269 | 95°C | 5 min | 1 |
| | | 95°C<br>55°C<br>72°C | 1 min<br>1 min<br>1.5 min | 40 |
| | | 72°C | 7 min | 1 |
| | | 4°C | ∞ | 1 |

*Gel Electrophoreses*

PCR amplifications were analyzed by gel electrophoresis. Molecular-biology grade agarose was added to lithium acetate (10 mM) with a 0.01% SYBR® Safe DNA Stain and melted by boiling in a microwave oven. The melted agarose was allowed to cool to about 50°C then cast in a gel mold. The lithium acetate buffer was chosen in place of the more common TBE buffer solution because of the lower conductivity of lithium acetate and its ability to be processed at higher speeds (5-30V/cm as compared to 5-10V/cm; Brody Et Al. 2004). Three milliliters of TrackIt™ 1Kb Plus DNA Ladder (Figure 4), was added into the first lane of each gel to allow size estimates of the PCR products.

One milliliter of each sample was mixed with eight milliliters of 10X BlueJuice™ gel loading buffer and pipetted into each of the corresponding gel wells. The apparatus containing the gel also contained a 1x lithium acetate buffer solution (without stain). Gels ran at 250-300V for between seven to twelve minutes. Finally, the gel was placed into a UV imaging system for DNA fragment visualization and image capture with a digital camera.

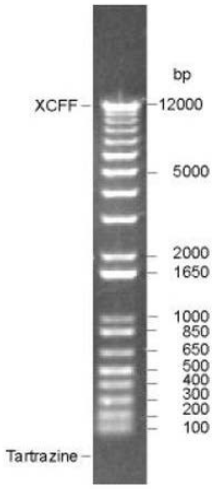


Fig. 7 TrackIt 1 Kb Plus DNA Ladder (Segal, 2008)

# CHAPTER 3

# RESULTS

## Surface Water Temperature

At each of the fifteen stations, a recording of the Surface Water Temperature (SWT) was logged. Figure 8 describes the monthly average temperatures of the surface water of these sampling stations. These temperature readings (°C) were taken from March through November, 2008.

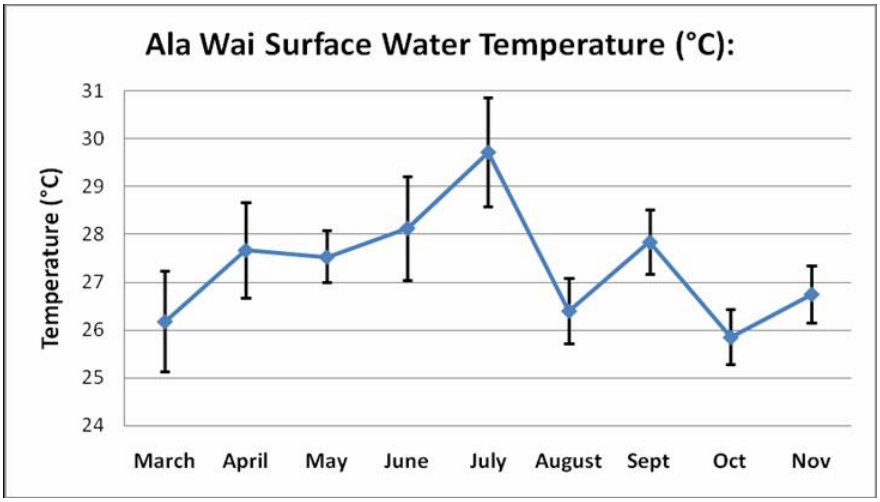


Fig. 8 Average Surface Water Temperature for All Stations

For the series-intensive month of July, a mean surface water temperature (SWT) was calculated. All temperatures were correlated to the samplings taken in the late morning to early afternoon hours. The peak surface water temperature recorded was

29.72°C in the month of July and the lowest surface water temperature recorded was 25.85°C in the month of October.

*Weekly Temperature and Salinity Variation*

Weekly temperature and salinity variations were recorded for Station One and Stations Five through Fifteen. Figure 9 graphically represents the observed temperature variations during each of the four sampling weeks. Even though the data fluctuates between stations and sampling days, it is important to note the temperature trends between each station and the correlation between temperature and stations distance from the harbor. The finite resolution of temperature may actually have ±0.25 degree variation due to thermometer size limitations. The following graph represents a generalization of each stations recorded temperature and not a linear geographical trend. The solid black line represents the overall trend from all four sampling days, showing a negative correlation between geographical distance and temperature.

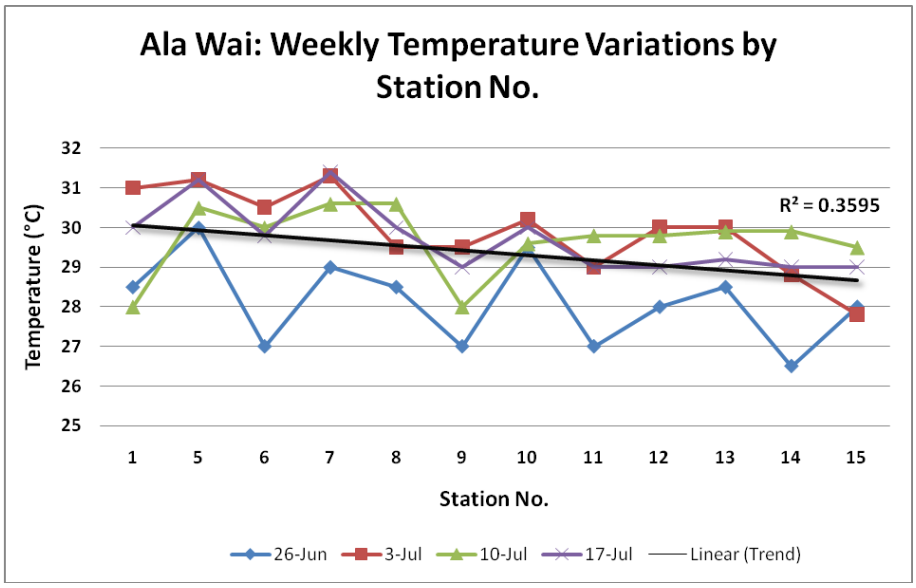

Fig. 9 Weekly Temperature Variation by Station

Similar to the temperature variations plot in Figure 9, Figure 10 graphically depicts the mean weekly salinity variations of Station One and Stations Five through Fifteen for the weeks of June 26th through July 17th, 2008. It is important to note that the plot does not infer a salinity trend between stations. Rather, the diagram gives an overview of the salinity variations according to the dates collected.

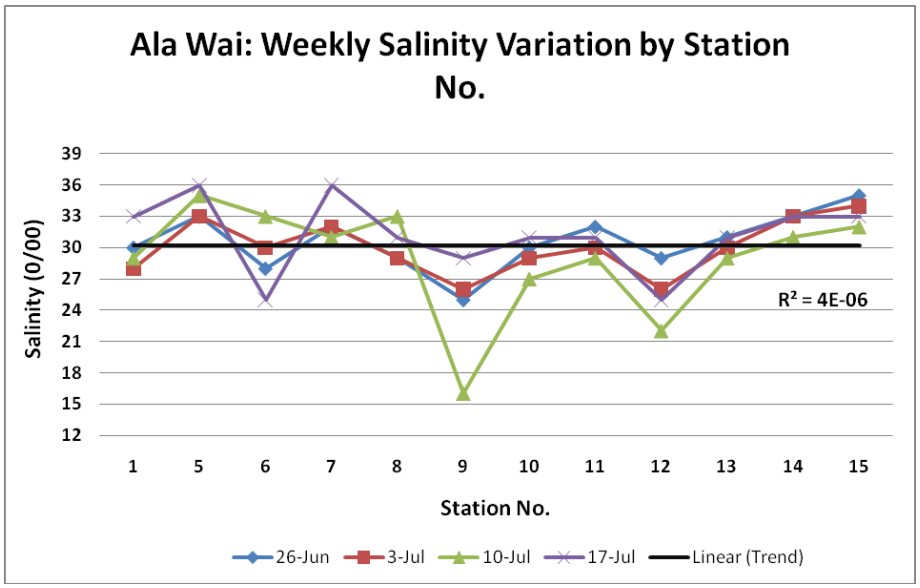


Fig. 10 Weekly Salinity Variation Verses Station

*Daily Temperature and Salinity Variation*

Unlike the previous weekly temperature and salinity figures, the daily temperature and salinity graphs depict only four different stations from July 10th through July 17th, 2008.

On average, Station Five was slightly warmer than the other three stations throughout the sampling week (figure 11). The irregularity the occurred on July 16th was because of an influx of fresh water that was due to the precipitation that occurred the previous night. Other than the slight irregularity, the daily temperature between each station remains relatively steady.

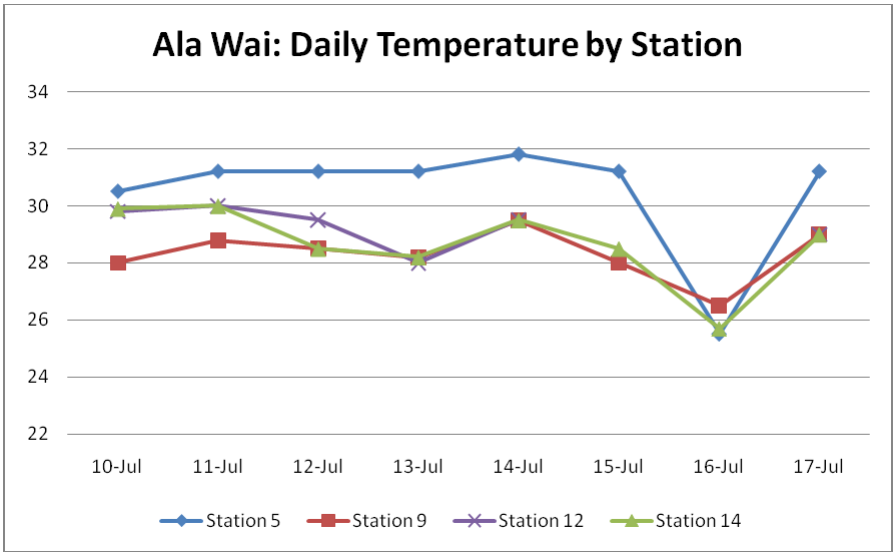


Fig. 11 Daily Temperature Variation by Station

Figure 12 illustrates daily variations of salinity measured at Stations Five, Nine, Twelve and Fourteen. Even though each salinity sample was taken at the same place for eight different days, the relative trends for each station are not all linear. The discontinuity see in Station Twelve from July 15th through 16th is due to an unforeseen sampling error.

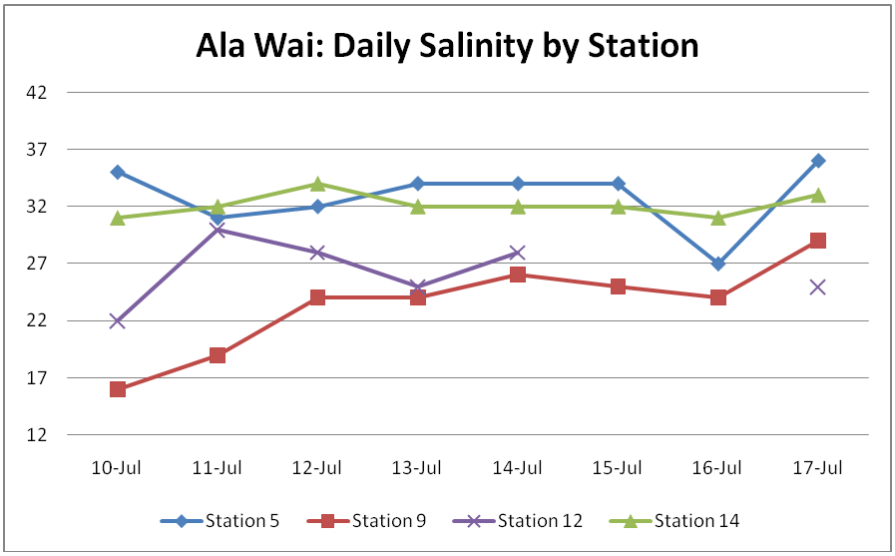


Fig. 11 Daily Salinity Variation by Station

*Hourly Temperature and Salinity Variation*

The daily temperature and salinity variation plots (Figures 13 and 14) helped identify two parallel components. First, hourly temperature varies with the time of the day and incoming solar radiation. And two, incoming tidal flux may have a direct role in salinity and temperature variation during some events.

A temporal-scale was collected from Station Five, Station Nine and Station Fourteen on July 15th and 16th, 2008. Beginning at 0600 on July 15th and ending at 0600 on July 16th, samples were collected from each site at three hour intervals. Figure 13 illustrates the temperature variances measured during the twenty-four hour period as compared with the tidal trends.

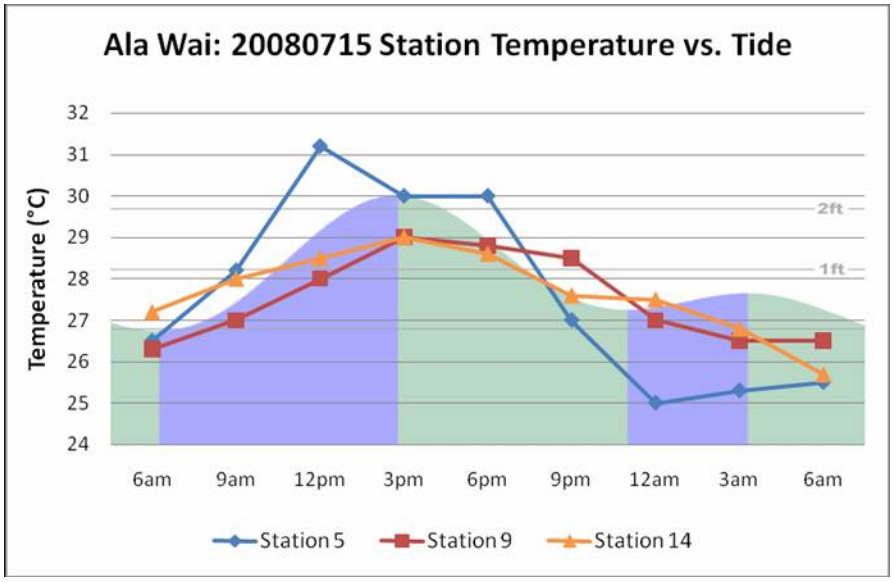


Fig. 12 Hourly Temperature Variations by Station

A comparison was made for Stations Five, Nine and Fourteen of the concentrations of salinity, the time of day, and the tidal flux (Figure 14). Even though the tidal flux graph is not to amplitude scale, important consideration should be noted of the salinity correlation. The hourly sampling event took place on July 15th through 16th, 2008. A sample from each of the three stations was logged every three hours, for a total of nine events. A critical irregularity, that will need further analysis, is the noticeable peak in salinity for station 5. The salinity at station 5 was higher than the salinity found in the open ocean water.

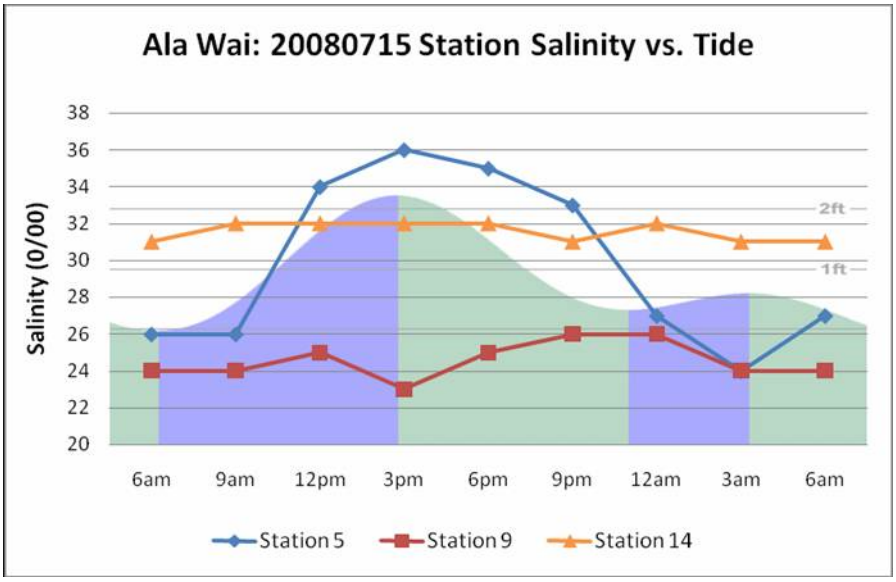


Fig. 13 Hourly Salinity Variations by Station

**Salinity**

The first step in understanding salinity variability in the Ala Wai Canal is to first identify the long term (seasonal) trends observed during the course of the experiment (Figure 9). The highest mean rate of salinity by month from March to November was recorded in July at 31.17 parts per thousand (ppt) and the lowest was during the October sampling event at 8.5 parts per thousand (ppt). The irregularity of the data recorded in October corresponds to the influx of precipitation within the Ala Wai watershed during the days prior to the October sampling. Figure 13 displays the average amount of rainfall in the region of the Ala Wai.

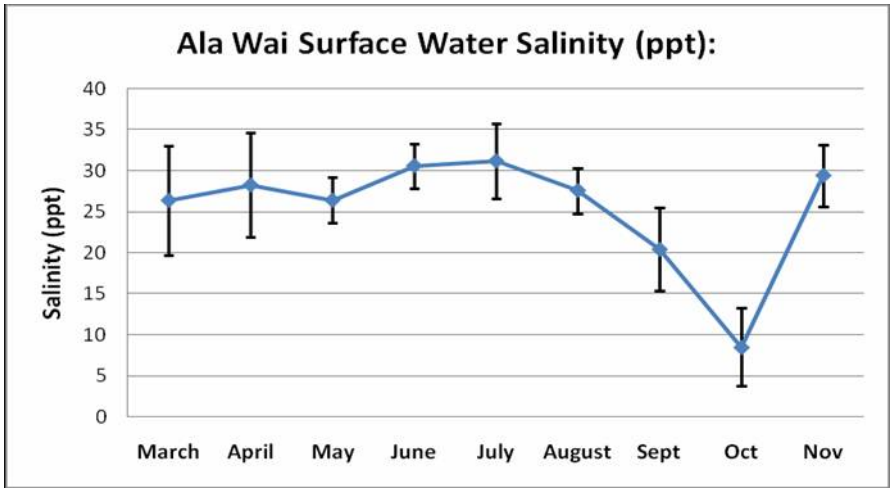


Fig. 14 Average Station Salinity by Month (March through November, 2008)

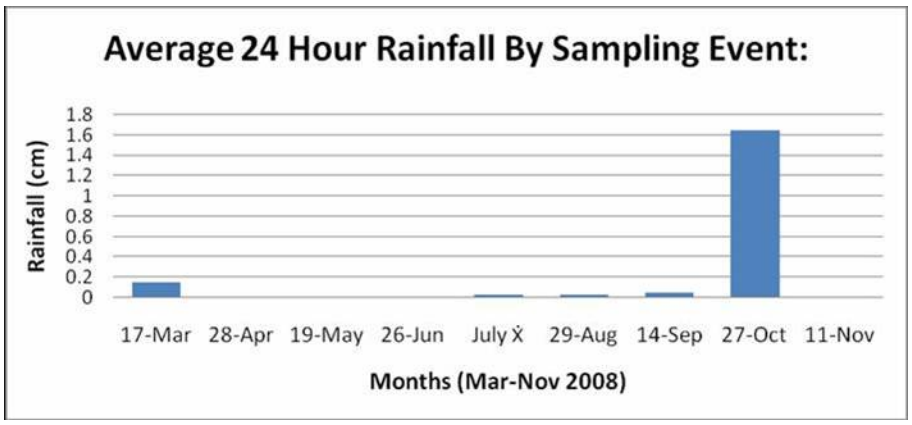


Fig. 15 Average 24 Hour Rainfall by Sampling Event[9]

[9] Each bar graph is the precipitation (rainfall) for the Waikiki area 24-hours prior to the sampling event that took place on each day. The month of July was averaged out for every sampling day.

*Weekly Salinity Variation*

Similar to the temperature variations plot in Figure 9, Figure 14 graphically depicts the mean weekly salinity variations of Station One and Stations Five through Fifteen for the weeks of June 26th through July 17th, 2008. It is important to note that the plot does not infer a salinity trend between stations. Rather, the diagram gives an overview of the salinity variations according to the dates collected.

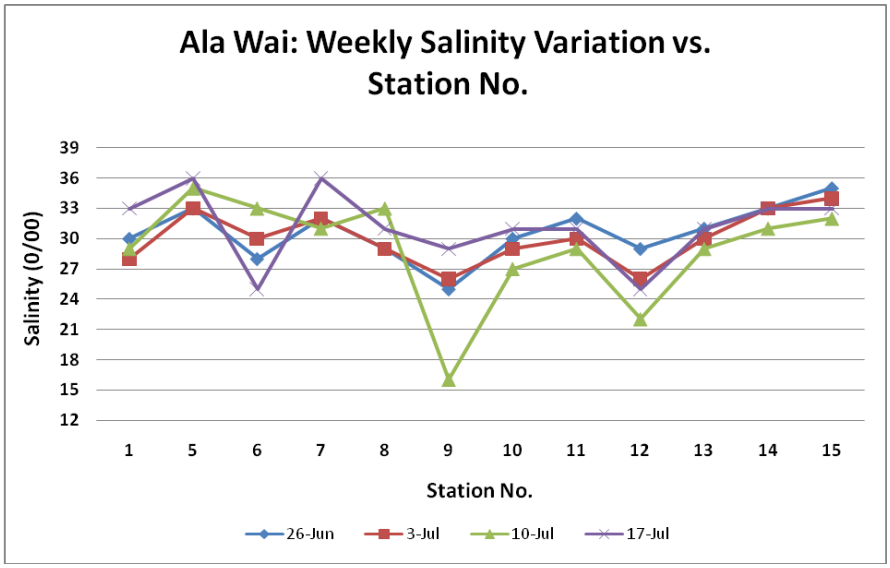


Fig. 16 Weekly Salinity Variation Verses Station

*Daily Salinity Variation*

Figure 15 illustrates daily variations of salinity measured at Stations Five, Nine, Twelve and Fourteen. Even though each salinity sample was taken at the same place for eight different days, the relative trends for each station are not all linear. The discontinuity see in Station Twelve from July 15th through 16th is due to an unforeseen sampling error.

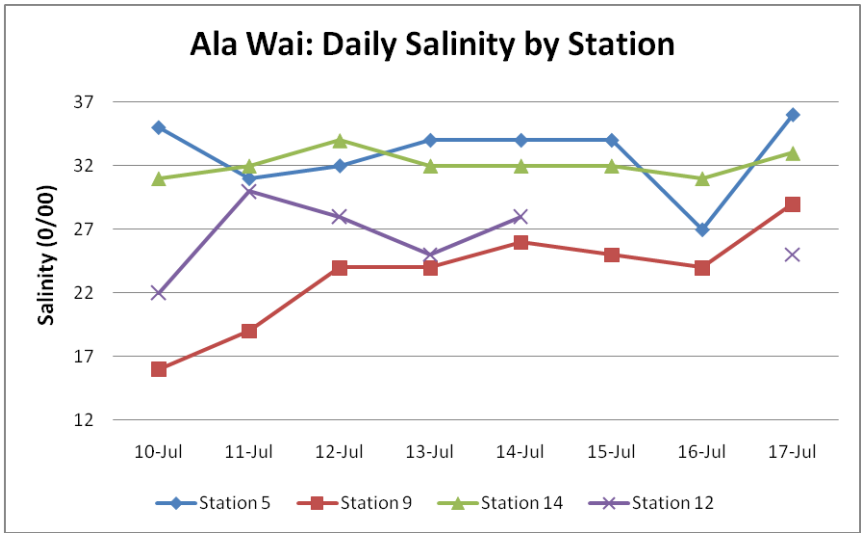


Fig. 17 Daily Salinity Variation by Station

*Hourly Salinity Variation*

A comparison was made for Stations Five, Nine and Fourteen of the concentrations of salinity, the time of day, and the tidal flux (Figure 16). Even though the tidal flux graph is not to amplitude scale, important consideration should be noted of the salinity correlation. The hourly sampling event took place on July 15th through 16th, 2008. A sample from each of the three stations was logged every three hours, for a total of nine events. A critical irregularity, that will need further analysis, is the noticeable peak in salinity for station 5. The salinity at station 5 was higher than the salinity found in the open ocean water.

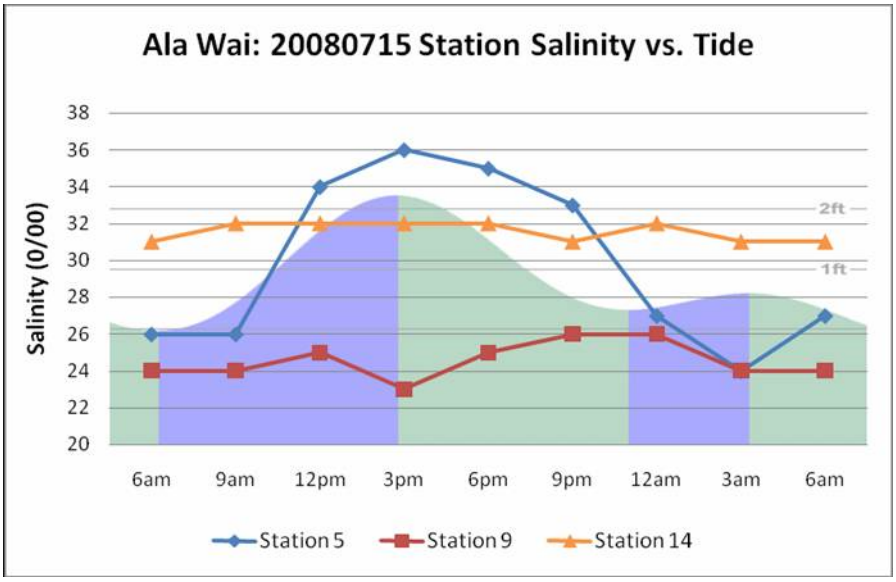


Fig. 18 Hourly Salinity Variations by Station

A scatter plot diagram of salinty parts per thousand (ppt) verses temperature (°C) is shown in Figure 17. The plot includes the one hundred and eighty temperature and salinity data points from the stations sampled throughout the entire scope of this study.

The linear trendline signifies the correlation between the increasing temperature and the salinity profiles. The r-value represents the proportion of the correlation coefficient, also represented as the linear relationship between the two series (temperature vs. salinity). The calculated r value for 181 recordings is 0.472, with a p value less than 0.01, which is well within the recognized means for statistical confirmation.

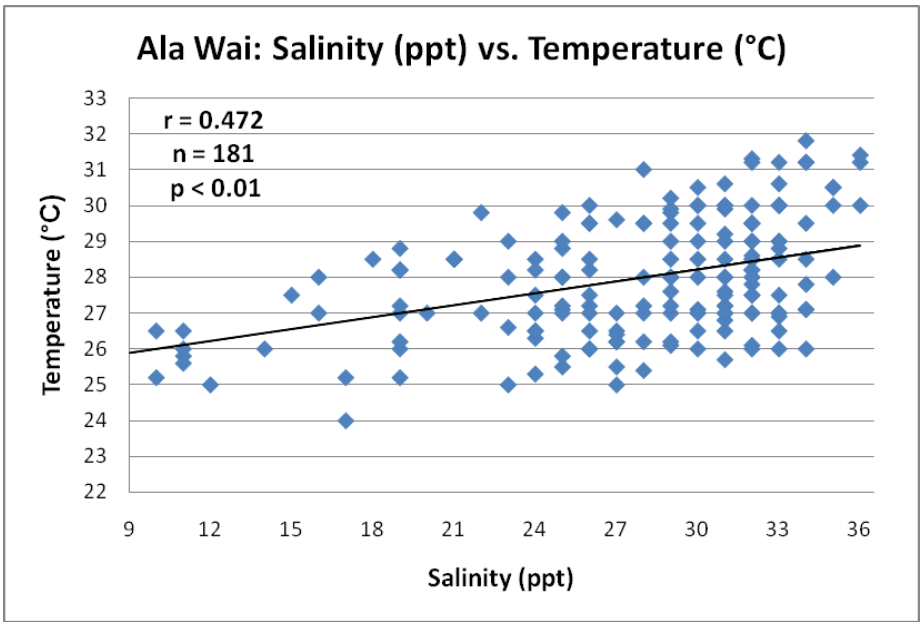


Fig. 19 Scatter Plot Diagram of Salinity (parts per thousand) Compared with Temperature for All Stations

## Tidal Fluctuations

The periodic and predictable tidal fluxes for the Ala Wai Canal are an obvious source of incoming sea water with elevated concentrations of salinity. The daily tidal markings for the Ala Wai Canal as related to each sampling event were recorded (Fig. 18), however, these samplings were not all collected at the same time of day. The relevancy of these tidal fluctuations will be described in the following chapter. During the first three sampling events, tidal flux was not a determining factor in collection date and time. However, later in the study, tidal flux and variation was the main determining factor for sampling day and time. Exceptions were made during daily and hourly sample. The ideal sampling time corresponded to a tidal flux around the low of the low daily tidal events. This ensured a relatively consistent marker for environmental variables. However, if water sampling was not possible during the low-low tidal time, the low of the high was substituted. The red and blue bar graph (Figure 18), summarizes the high and low tides from each sampling day.

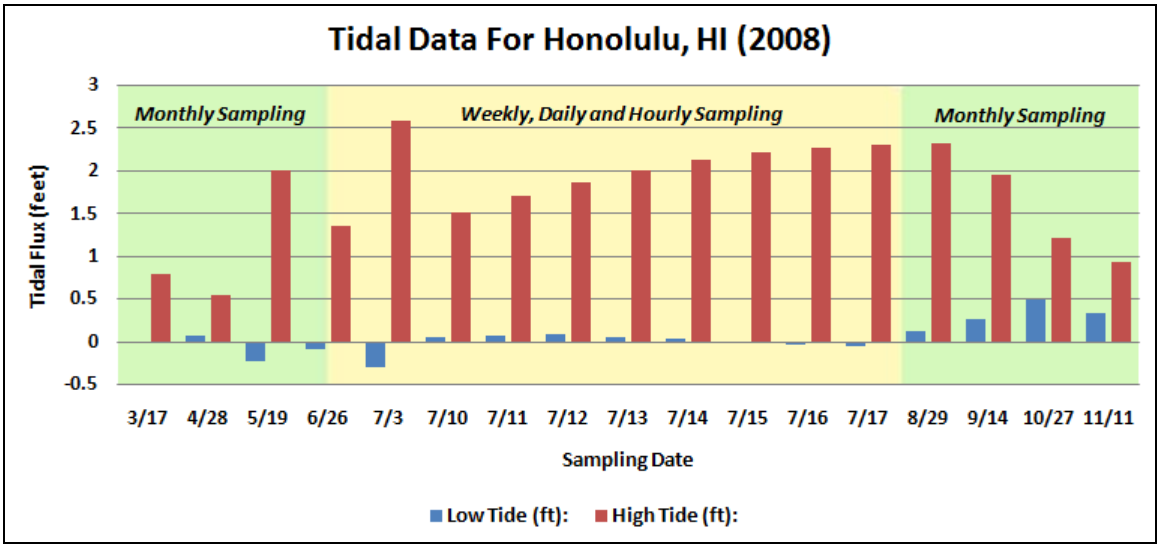


Fig. 20 Summary of Tidal Data from Each Sampling Event, March through November 2008

## *Vibrio* Selective CFU's

*Vibrio-selective Media: Thiosulfate Citrate Bile Sucrose Agar*

The highest average number of green-colored Colony-Forming Units (CFU) was recorded at Station Three at approximately 70.1 CFU's (fig 19). The lowest mean Colony-Forming Units (CFU) were recorded Station Ten, within a range of approximately 1.2 CFU's.

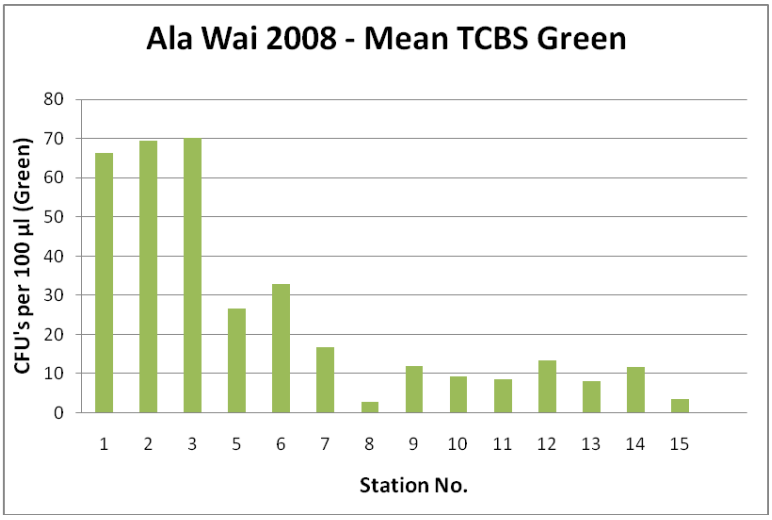


Fig. 21 Average Green-Colored Colony-Forming Units from Three Sampling Events

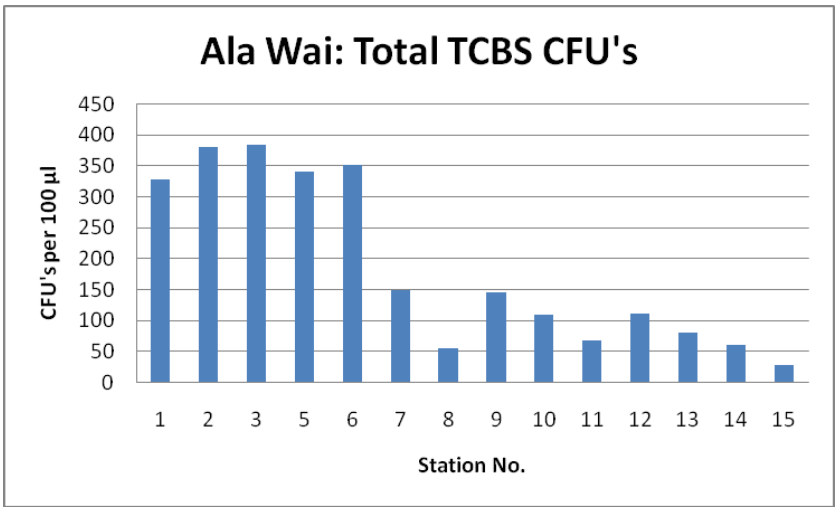


Fig. 22 Total Counted Colony-Forming Units per 0.1ml Thiosulfate Citrate Bile Sucrose Agar for Each Station

*Vibrio-selective Media: CHROMagar Vibrio*

The highest mean mauve-colored Colony-Forming Units (CFU) recorded by station was for Station Two at approximately 10.75 CFU's (Fig. 21). The lowest was at Stations One and Three, with a total of 7.5 mauve-colored CFU's per 0.1ml. The patterns in total vibrio abundance showed similar spatial trends as was observed for the putative V. parahaemolyticus (Fig. 22).

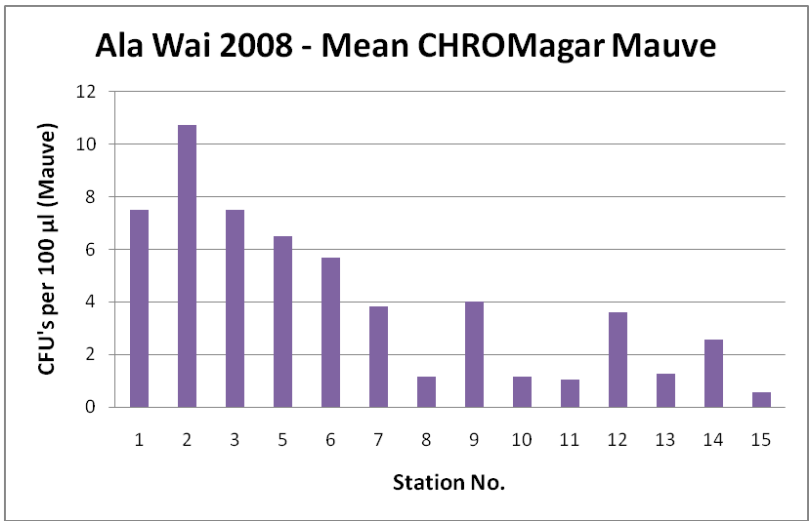


Fig. 23 Average Mauve-Colored Colony-Forming Units per 0.1ml for All Stations

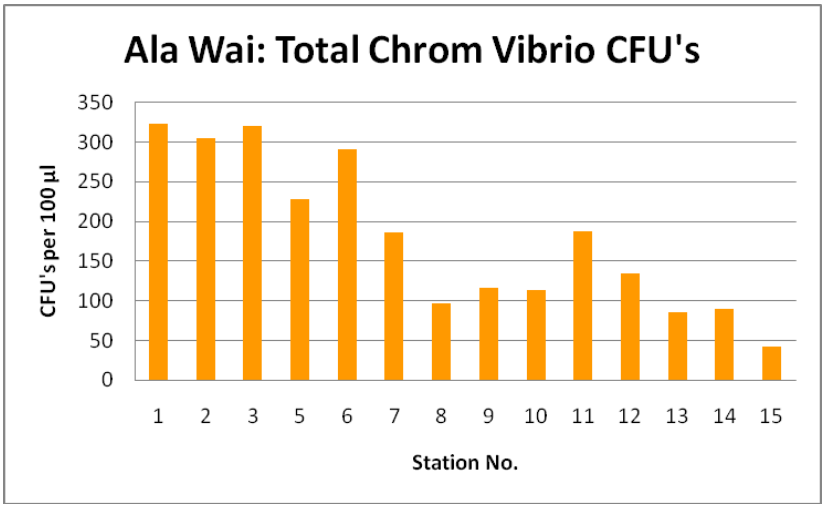


Fig. 24 Total Counted Colony-Forming Units per 0.1ml CHROMagar *Vibrio*

*Comparative Overview of Plate Isolate Results*

On average for all stations at three sampling times (March, June and September), the percentage all colonies on TCBS that were green was 39% and the percentage of all colonies on CV Vibrio that were mauve was 6% (Fig. 23). The total CFU counts on

TCBS and CV were highly correlated (r = 0.88, n = 40, p <0.01; Fig. 24), althogh CFUs on CV tended to be higher than on TCBS when the totals were high. Since *Vibrio parahaemolyticus* is reflected in the mauve-colored Colony-Forming Units on CV *Vibrio* media and in the green-colored Colony-Forming Units on Thiosulfate Citrate Bile Sucrose (TCBS) media agar, this study will not focus any further on yellow CFU's from TCBS or White and Blue CFU's from CV.

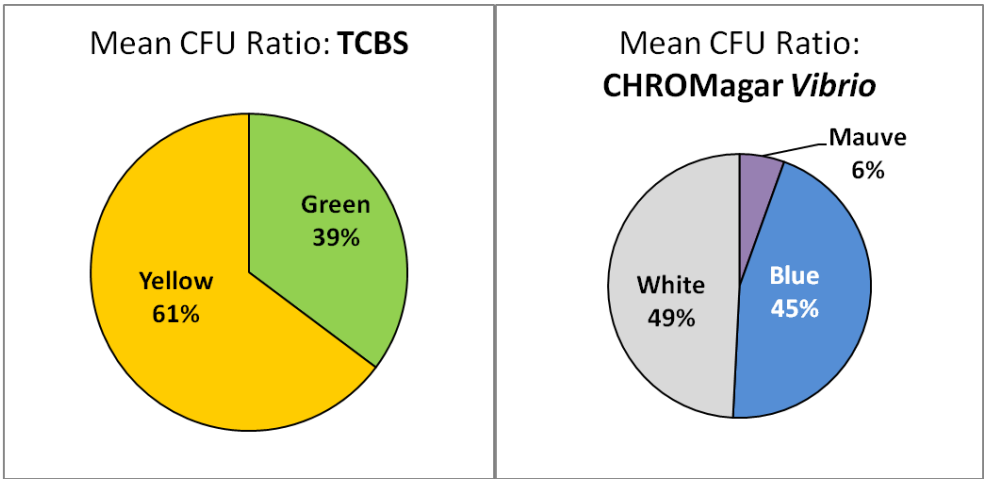


Fig. 25 Mean Colony-Forming Unit Color-Ratio for Both Thiosulfate Citrate Bile Sucrose Agar and CHROMagar *Vibrio* Media

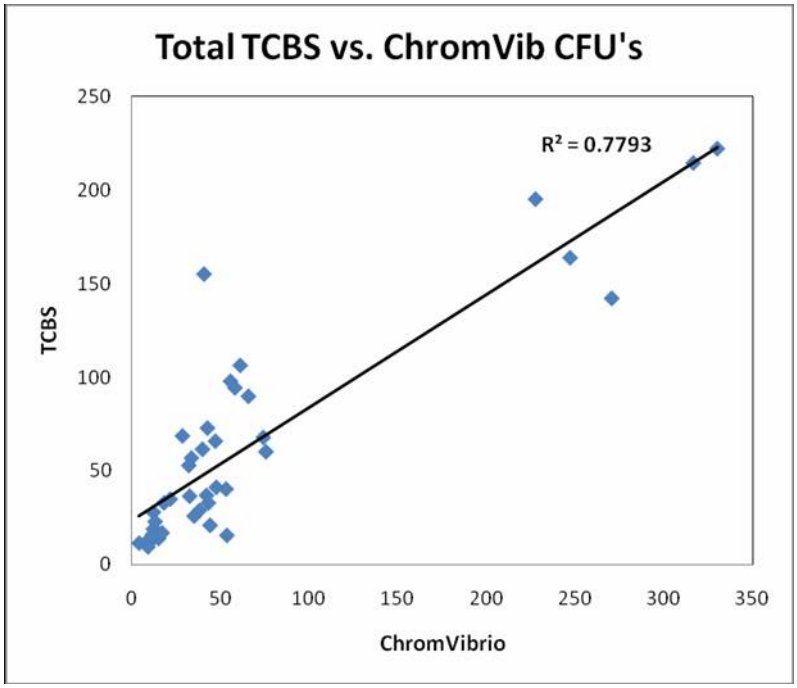


Fig. 26 Total TCBS and Chrom *Vibrio* Count Comparison

*CFUs Correlation to Salinity and Temperature*

To further understand the effects of salinity and temperature on *Vibrio parahaemolyticus*, several plots were constructed to demonstrate any possible correlations. The scatter-plot diagrams of both Thiosulfate Citrate Bile Sucrose (TCBS) agar and CV Media are indicated according to the corresponding temperature and salinity values (Figures 25-28). Even though an isolate might appear to represent a specific *Vibrio* species, a PCR analysis was required to confirm its identity.

There was no consistent relationship between the number of green colonies and temperature across the entire temperature range (24 to 32°C; Figure 25). However, highest values tended to be found in the middle of this range (27 to 28.5°C).

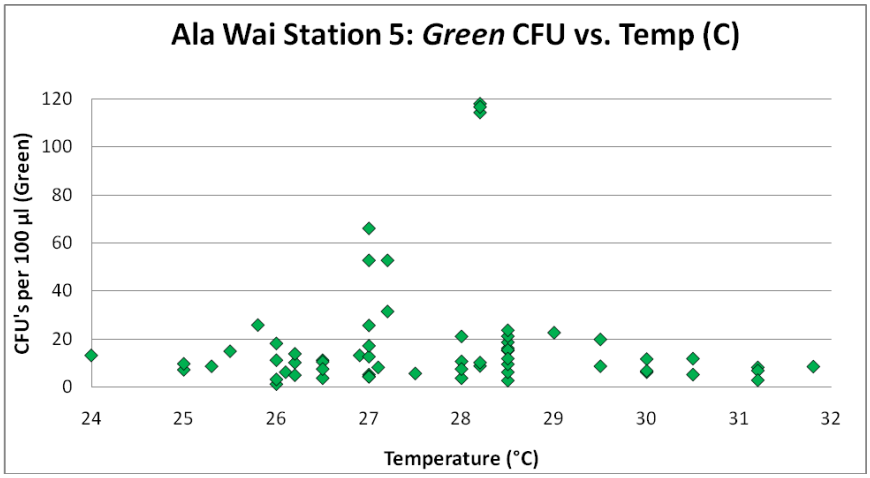


Fig. 27 Scatter-plot Diagram of Temperature Compared with *Green* Isolates (Thiosulfate Citrate Bile Sucrose Agar) CFU's for All Sampling Events

A similar pattern was observed in the relationship between salinity and *V. parahaemolyticus* abundance, with lowest values observed at the low (10 to 18 ppt) and high (23 to 36 ppt) ends of the salinity range (Fig. 26). Higher values were found in the middle of the range (19-22 ppt).

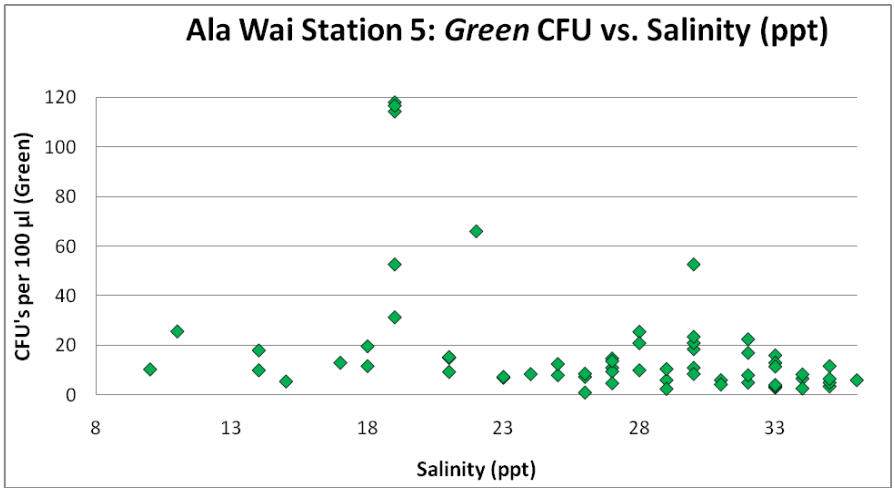


Fig. 28 Scatter-plot Diagram of Salinity Compared with Green Isolates. (Green, Thiosulfate Citrate Bile Sucrose Agar) CFU's for all Sampling Events

As seen with the green colonies on TCBS, there were no consistent trends between the abundance of mauve-colored CFUs and either temperature or salinity across their entire ranges (Figs. 27-28). However, highest values were observed at moderate temperatures (27-28 °C) and salinities (18-23 ppt).

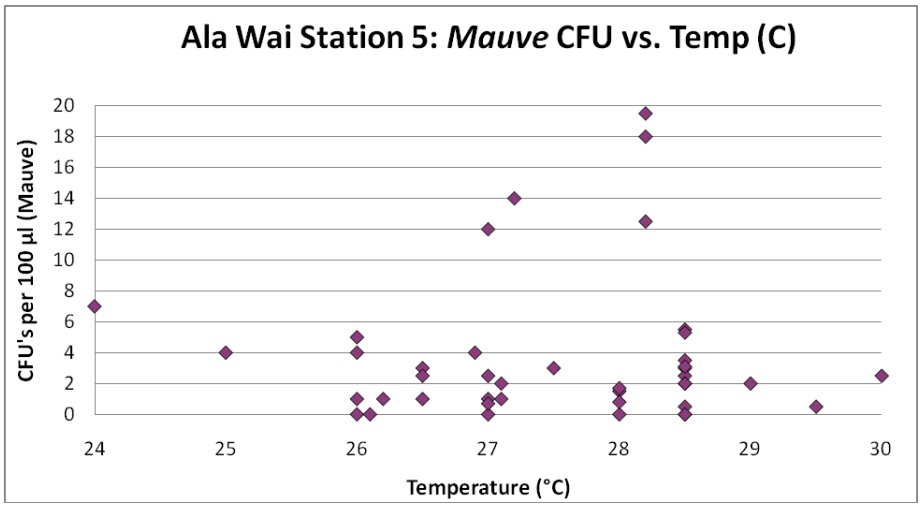


Fig. 29 Temperature Compared with *Vibrio parahaemolyticus* (Mauve, CV Media) CFU's

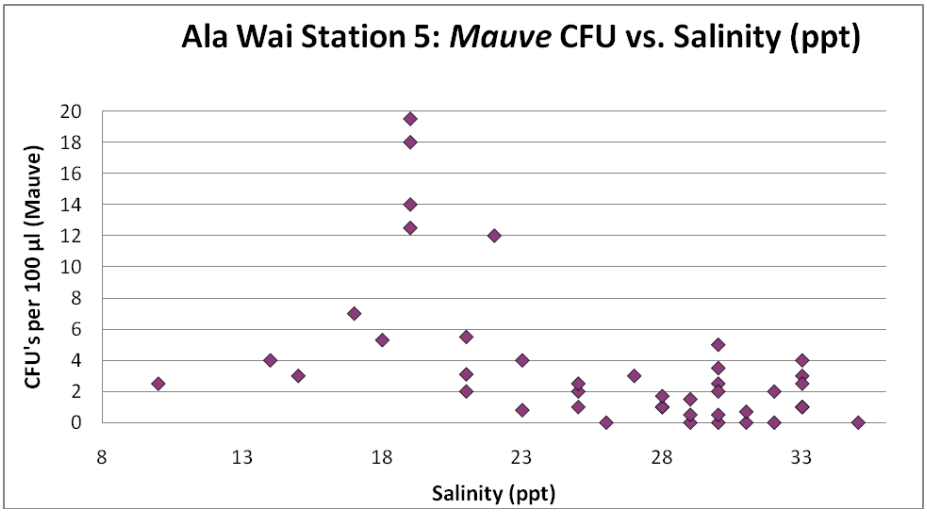


Fig. 30 Salinity Compared with *Vibrio parahaemolyticus* (Mauve, CV Media) CFU's

A further analysis of the data compiled from the mauve CFU vs. salinity plot (Figure 29), shows both a positive and then negative trend at two distinctive salinity ranges. These salinity ranges were then broken up into two separate graphs with corresponding salinity ranges (Figures 29 and 30. Figure 29 shows a strong positive correlation between mauve CFU's and salinity between the salinities of nine through nineteen. Conversely, there is a relatively moderate negative correlation that was plotted in Figure 30, between salinities nineteen through thirty-six. To gain a linear plot, the log value of each mauve CFU was first calculated then graphed.

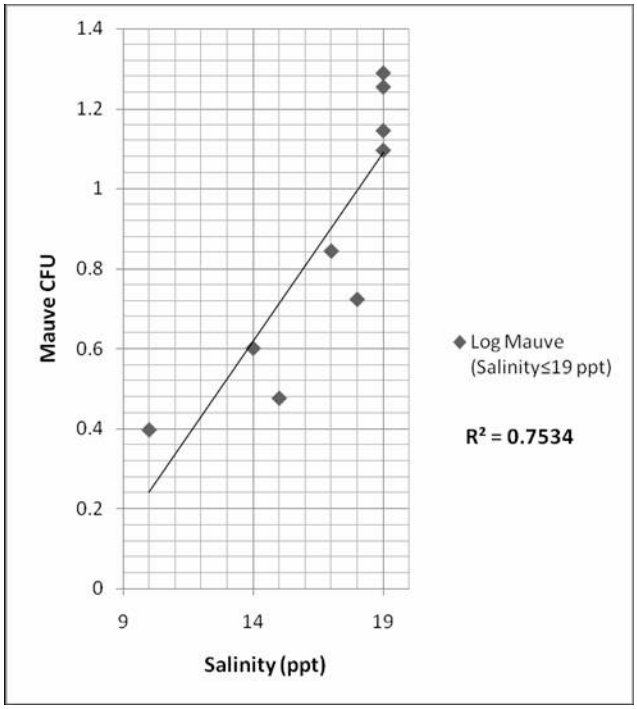


Fig. 31 Salinity (9 through 19 ppt) versus Log Mauve CFU Values

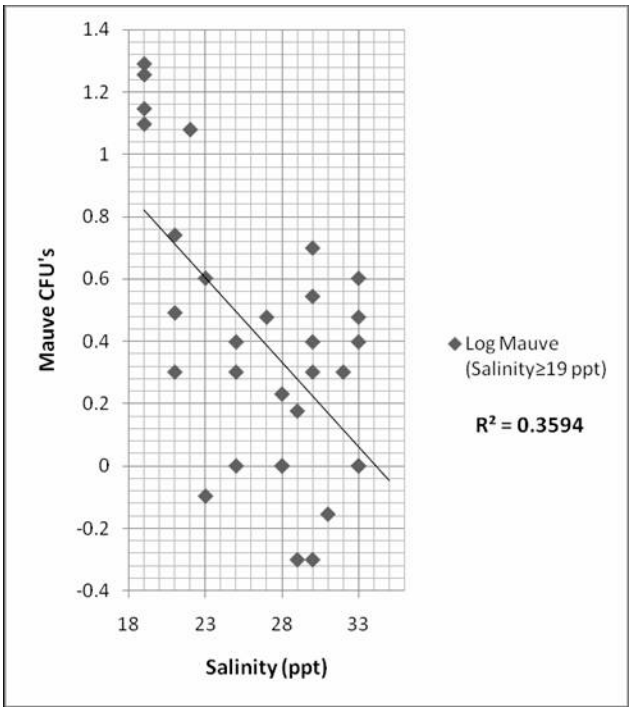


Fig. 32 Salinity (19 through 36 ppt) versus Log Mauve CFU Values

The abundance of green and mauve colored colonies varied among sites within the Ala Wai canal (Figs. 31-32). The twelve bubbles are positioned at the location of each of the sample sites. The relative abundance of CFU's at each site is proportional to the area of the circle.


Fig. 33 Geographical Representation of Green-colored CFUs
(*Vibrio parahaemolyticus*, Thiosulfate Citrate Bile Sucrose Agar)
Along the Ala Wai Canal, Honolulu. Area of the bubble is proportional to the relative abundance.

Fig. 34 Geographical Representation of All Isolated Mauve-colored (*Vibrio parahaemolyticus*, CV Media) CFU's Along the Ala Wai Canal, Honolulu. Area of the bubble is proportional to the relative abundance.

## DNA Analysis

Upon purification and isolation of the putative *V. parahaemolyticus* , their identities were confirmed by PCR analysis. Only isolates from one event (September 2008 sampling) were analyzed due to the limited time available to complete this portion of the project.

### *16S rRNA PCR*

Of the isolates tested , 77.5% amplified successfully with the16S rRNA gene primers (Table 8). An example of the results of this assay are shown in Figure 33 . The 16S rRNA PCR product length is 1492 base pairs (bp), and can be seen in rows two through seven. It is important to note that during the process, rows two and six showed some non-specific amplification.

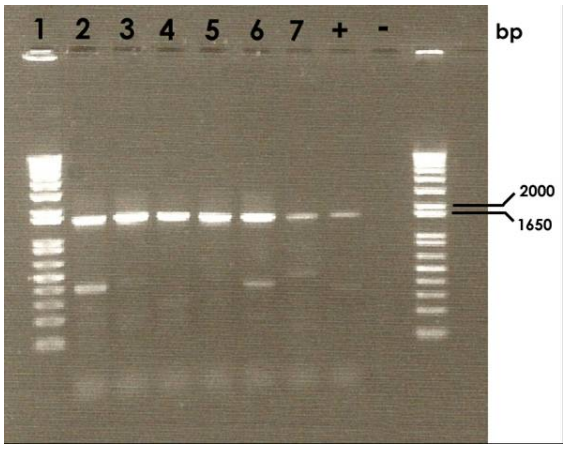


Fig. 35 Gel-electrophoresis Assay of 16S rRNA PCR

*Vibrio-Specific Gene(s) Analysis*

After successful completion of the 16S rRNA gene identification process, the next step was to quantify the abundance of positive *V. parahaemolyticus* by *tlh* PCR amplification. Figure 34 illustrates a gel in which *tlh* was successfully amplified for the September sampling event. The corresponding length of the *tlh* gene is 450 bp as shown by the bright bands located in Figure 34.

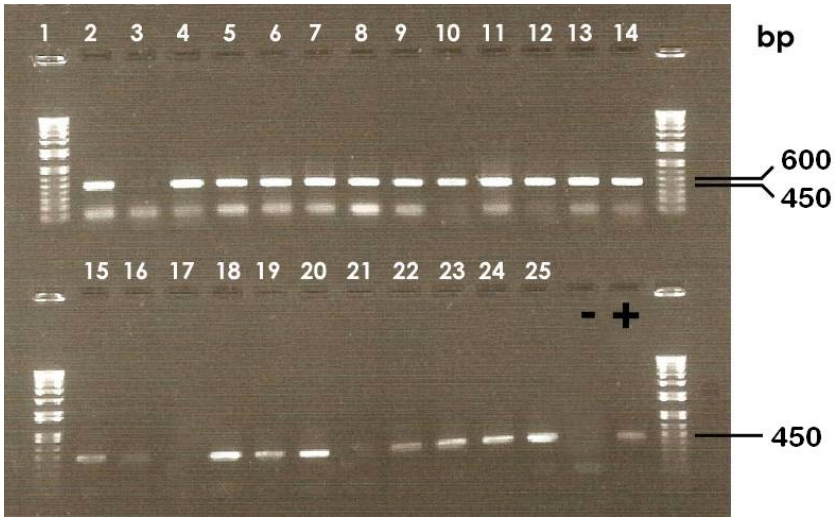


Fig. 36 Agarose Gel Electrophoresis Showing from *tlh* Amplification

*Virulence Gene(s) Analysis*

A final analysis was conducted in the amplification of the *tdh* PCR gene. Figure 35 shows the results of the *tdh* gel electrophoresis. As shown in Table 8, out of the total thirty-one isolates inserted into the PCR, no products of the correct size were amplified. Rows six, thirteen, twenty-two and twenty-nine revealed forms of non-specific amplification.

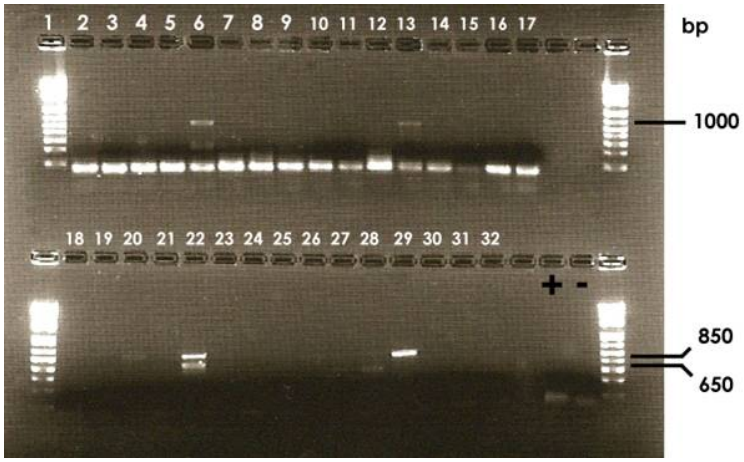


Fig. 37 Agarose Gel Electrophoresis Results from *tdh* Amplification

Table 8 is a statistical breakdown of the results from each of the three PCR assays used to analyze the September sampling event. Only the successful *tlh* genes were then used in the *tdh* PCR analysis.

Table 4 Statistical Results from Each PCR Analysis (16S rRNA, *tlh* and *tdh*) for September, 2008. Only samples positive for 16S rRNA amplification were used n the calculation of percent *tlh*- or *tdh*-positive.

| PCR Assay Type | Total Samples Used | Total Positive | % Total |
|---|---|---|---|
| 16S rRNA | 48 | 36 | 75 |
| *tlh* | 36 | 26 | 72 |
| *tdh* | 31 | 0 | 0 |

Table 9 details the number of samples evaluated, the media growth type that was used, and the station numbers of the samples retrieved. The presence of *Vibrio parahaemolyticus* was confirmed within the Ala Wai Canal.

Table 5 Results from PCR and DNA Arrya Hybridization of Ala Wai Water Samples, September, 2008

| Sample | ID No. | Station No. | Media Growth Type | *V. parahaemolyticus* | | |
|---|---|---|---|---|---|---|
| | | | | 16s rRNA | *tlh* | *tdh* |
| 1 | V86A3P | 1 | CV | + | + | - |
| 2 | V86A5P | 2 | CV | + | + | - |
| 3 | V86B1P | 2 | CV | + | + | - |
| 4 | V86B3P | 3 | CV | + | + | - |
| 5 | V86B4P | 3 | CV | + | + | - |
| 6 | V86B5P | 3 | CV | + | + | - |
| 7 | V86C2P | 4 | CV | + | + | - |
| 8 | V86C3P | 4 | CV | + | + | - |
| 9 | V86C5P | 5 | CV | + | + | - |
| 10 | V86D1P | 5 | CV | + | + | - |
| 11 | V86D5P | 6 | CV | + | + | - |
| 12 | V86D6P | 6 | CV | + | + | - |
| 13 | V86E1P | 7 | CV | + | + | - |
| 14 | V86E2P | 7 | CV | + | + | - |
| 15 | V86E4P | 7 | CV | + | + | - |
| 16 | V86F5P | 9 | CV | + | + | - |
| 17 | V87A2P | 10 | CV | + | + | - |
| 18 | V87A5P | 11 | CV | + | + | - |
| 19 | V87B3P | 12 | CV | + | + | - |
| 20 | V87B4P | 12 | CV | + | + | - |
| 21 | V87C1P | 13 | CV | + | + | - |
| 22 | V87C2P | 13 | CV | + | + | - |
| 23 | V87C6P | 14 | CV | + | + | - |
| 24 | V87D2P | 14 | CV | + | + | - |
| 25 | V88A1P | 1 | CV | + | + | - |
| 26 | V88B4P | 3 | CV | - | + | - |
| 27 | V89A5P | 11 | CV | - | + | - |
| 28 | V91C2P | 4 | TCBS | - | + | - |
| 29 | V91C3P | 4 | TCBS | - | + | - |
| 30 | V91F3P | 9 | TCBS | - | + | - |
| 31 | V92B1 | 11 | TCBS | - | + | - |

# CHAPTER 4

# DISCUSSION

## Overview of Relationships, Trends and Generalizations

Previous studies have demonstrated strong relationships between *Vibrio* abundance and environmental conditions such as salinity, temperature and attachment to planktonic organisms (Tamplin, 1990; also Heidelberg, 2002). Therefore, the researcher of this thesis will offer no further discussion about the relationship between planktonic organisms and environmental parameters. However, it is the intention of this writer to discuss the relationship of the abundance of *Vibrio parahaemolyticus* in the Ala Wai Canal to the conditions of temperature, salinity, *Vibrio*-selective colony-forming units, and the microbiological data derived from Polymerase Chain Reaction (PCR) for three separate identification genes.

### *Tidal Variability and Impact on Temperature and Salinity*

The Ala Wai Canal has three primary sources of water flux: fresh water from incoming streams, atmoshperic deposition in the form of direct rain, and saline-rich sea water. The Ala Wai estuary has a semidiurnal tidal characteristic (i.e. two high water and two low water tides each day). This frequent and somewhat predictable input of salty sea water affects the daily temperature and salinity concentrations of the waters of the Ala Wai. Characteristic fluctuation in temperature and salinity was recorded during the twenty-four hour sampling event on July 15$^{th}$ and 16$^{th}$, 2008. And even though most

stations indicated some variation, Station Five stands alone as being the site which had the greatest difference in both temperature and salinity (T/S).

This was particularly interesting because unlike Stations Nine and Fourteen, Station Five was located the furthest from the mouth of the Ala Wai Canal as it empties into the harbor. One might have assumed that Station Five would have had the lowest T/S profile. However, relative to both variables it seems as if the opposite is true. Perhaps the influx of saline water at Station Five was caused by super-saline non-stratified waters that became exposed to the near-surface waters during high tides or elevated evaporation rates.

Besides the uncharacteristic change in temperature and salinity (T/S) profile from station 5, the others stations do show moderate to significant correlation with tidal influx. It is important to note that for the twenty-hour collection event, no precipitation was recorded in the Ala Wai area. Another key point of interest is that fact that the increase in sea surface temperature could, but not likely be caused, mainly due to incoming solar radiation. Even though all stations experienced an increase in temperature during the peak radiation times of the day, the immediate drop in temperatures in the later afternoon and evening hours shows that no absorbed solar heat was conserved.

### *Sea Surface Salinity Variability in Proportion to Temperature*

The first observation is the direct relationship between salinity and temperature can be seen in Figure 17. The plot diagram shows that with an increase in temperature there is a strong, though not definite, correlation to an increase in temperature. This correlation is important to discuss because of the nature of *Vibrio* species and their inherent ability to be positively influence by both salinity and temperature levels. The

reason why there is a positive correlation between the waters salinity and temperature is a little more cumbersome to digest. Several facts may attribute the positive correlation in the Ala Wai Canal. A likely attributing factor towards the positive correlation could be the face that the majority of all temperature samples were collected during daylight hours, with highest incoming solar radiation. This temperature inconsistency would skew the data towards a higher than normal mean temperature vs. salinity. Another important consideration is that on a global basis, not all T/S profiles show a positive correlation.

There are three main sources of freshwater stream flow into the canal, Manoa stream, Makiki stream and numerous artery storm drains, the largest of which is at Stations One through Six. An observed long-term trend between salinity and site location. As predicted over the entire scheme of our sampling event, Stations One, Nine and Twelve all showed significant average salinities lower than the average and well below the normal sea water levels. However, it is important to realize that as stated before, the bathymetry of the canal and possible unforeseen irregularities could attribute to the changes in station salinity.

### *CFU Growth and Possible Vibrio parahaemolyticus Abundance*

Analysis from CFU data show that spatial and temporal variability does exists in the Ala Wai Canal and CFU abundance may be dependent on environmental parameters such as temperature and salinity. Geographical variance in mauve and green CFU's varies along the canal, as shown in the two maps. Furthermore, in comparison with other similar studies, we can observe that there exists a threshold for peak of abundance for both salinity and temperature. However, the observed peak ranges in both salinity and

temperature, may be attributed to other unknown environmental influences such as nutrient input and chlorophyll *a*.

Statistical analysis of temperature vs. CFU growth showed relatively weak correlations, on both TCBS and CV media. However, a stronger correlation was observed when CFU's were plotted against salinity. The CFU vs. Salinity plot (figure 28) showed a positive correlation between salinities below twenty and above eight ppt. When plotted on a log scale, a strong positive and then negative correlation was established (figure 29 and 30).

Understanding the spatial distribution and temporal patterns of temperature and salinity in the Ala Wai Canal will aid with the overall understanding of where and when *Vibrio parahaemolyticus* may occur in significant abundance. However, confirmation of their relative harm to human health is still not yet understood for this particular estuary. This then leads to the next section describing the confirmed presence of *Vibrio parahaemolyticus* and possible virulent determinants.

### *Gene Expression of Isolated Vibrio parahaemolyticus*

The presence of high levels of *Vibrio parahaemolyticus* in the Ala Wai estuarine system may pose a specific health threat to the individuals that are exposed to contaminated waters. Understanding weather the water in the Ala Wai contains *Vibrio parahaemolyticus* is one area of interest, however, as previously stated, not all strains are truly pathogenic in nature. Previous studies has shown that in order to identify a potentially pathogenic strain, it is necessary to target multiple genes for PCR amplification. In this study, species specific *tlh*, and pathogen strain-specific *tdh* were selected for amplification and detection. In this study, we established a DNA assay that

enabled us to confirm post-PCR detection of the naturally occurring bacterium, *Vibrio parahaemolyticus* and see if there is a presence of pathogens specifically harmful to human health. Our data demonstrated that a large abundance of isolated mauve-colored CFU's were in-fact true positives for *Vibrio parahaemolyticus*. However, we did not identify any strains with the *tdh* gene. We did, however, analyze relatively few isolates so strains with this virulence-associated gene may be still be present in this environment.

# CHAPTER 5

# CONCLUSIONS

*Vibrio parahaemolyticus* is a naturally occurring bacteria in marine and estuarine environments around the world. This pathogenic species can negatively affect human health in almost every region of the globe. *Vibrio parahaemolyticus* populations are capable of rapid adaptation in response to changing environmental conditions, making them dynamic over short term and seasonal scales. Temperature, vertical mixing, tidal flushing, climate, precipitation and nutrient loading can change the estuarine environment and subsequently alter the microbial community structure of the bacterium, affecting estuarine water quality and public health.

The major advances in recent years in the knowledge of environmental factors affecting *vibrio* viability, persistence, virulence, and transmission to humans have allowed integration of these studies with those derived from genome analysis and gene expression, prerequisite to control and prevention strategies ensuring maximum protection of human health.

In conclusion, our results demonstrate temporal and spatial variations do exist in the densities of pathogenic *V. parahaemolyticus* in the Ala Wai canal and harbor. However, significant relationships between *V. parahaemolyticus* abundance and surface water salinity and temperature were not observed when considering the data set in its entirety. This could be explained by the fact that the optimal temperature and salinity for

growth of this species lie in the middle of the ranges sampled and that other factors may equally important in controlling their abundance.

Another important aspect of *V. parahaemolyticus* risk assessment is the fact that our results did not show any positive hits for virulence-associated genes. This may suggest that virulent strains are not abundant in the canal. These observations of *Vibrio parahaemolyticus* dynamics in the Ala Wai Canal and watershed may provide evidence that populations interact with environmental influences in the surface waters. Even though the State of Hawai`i has the highest *per captia* Vibrio-related infection rate in the U.S., the actual number of *V. parahaemolyticus* infections is minimal. This information may prove critical for future models of estuarine bacterial dynamics and distribution of pathogenic bacteria.

# CHAPTER 6

# RECOMMENDATIONS

In the spring of 2009, a comparative analysis of *Vibrio vulnificus* will be analyzed using similar techniques. However, several incomplete analyses may help shed more light on the influences of overall *Vibrio* abundance in the Ala Wai watershed. They include, total organic carbon (TOC), Chlorophyll *a*, dissolved iron, Sterivex filtration analysis, nutrients and particular organic carbon (POC).

The next step would be to use the analyzed and quantifiable data and create models useful to predicting the possible abundance of *vibrio* in the Ala Wai estuarine system and other tropical coastal systems of the world.

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