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RAPID RESPONSE: APPLICATION OF A qPCR-BASED TEST FOR ENTEROCOCCI AND HUMAN-ASSOCIATED *BACTEROIDES* AS A RAPID BEACH MANAGEMENT TOOL IN HAWAI'I

A THESIS SUBMITED TO THE GRADUATE DIVISION OF THE UNIVERSITY OF HAWAI'I IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE

IN

CIVIL ENGINEERING

DECEMBER 2017

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ACKNOWLEDGEMENTS

I would like to thank Augustine Sandoval and Anthony Shangirn from Hawai'i Kai wastewater treatment plant, Robert Choate from Honouliuli wastewater treatment plant and Dr. Gayatri Vithanage from Sand Island wastewater treatment plant for the help with wastewater sample collection. I would also like to thank the help from Surfrider Foundation volunteers Rafael Bergstrom, Mike Billet, Sarah McSkim, Daniel Jenkins, Matt Moore, Lena Diaz, Risa Oram and Kelsey Barden for the help in sample collection from Ala Wai Canal, Ala Moana Bowls, Kahanamoku Beach and Lagoon Oahu. I would like to thank my thesis committee members Dr. Moravcik, Dr. Babcock and Dr. Yan for helping me with ideas and editing in this paper. I especially would like to thank my mentor and friend Marek Kirs for all his time and effort during the development of this project. Thank you for your orientation and for believing in my potential and work. Lastly, I would like to thank my family for giving me support since I moved to the United States and for encouraging me to stay for my Master Degree.

ABSTRACT

Climate change is projected to increase the risk of loss for people, assets, economies and ecosystems, as extreme weather events, such as tropical storms and hurricanes, will increase in number and intensity. Changing climate and urbanization will alter inputs of fecal bacteria in the environment which can compromise the health of Hawai'i residents and visitors. One problem we face regarding fecal bacteria in the environment is the inadequacy of the existing methods to detect their presence and numbers quickly enough to be able to warn swimmers about contaminated water. Currently used culture-based microbial detection methods take a minimum of 24 hours to complete, while newer rapid molecular methods, can be completed in a few hours. Hawai'i is extremely well suited for the application of these rapid methods due to the small land mass, high population density and high numbers of visitors to the islands. Unfortunately, no validated and ready to use in Hawai'i rapid method exists. Our preliminary studies identified several critical problems with the rapid methods that are specific to Hawai'i and likely to other tropical regions. One of the problems is the high background levels of enterococci from non-human sources found in Hawai'i's nearshore waters that could lead to beach warnings being posted when no actual sewage contamination, and related health hazard, exists. This could negatively impact public perception of Hawaii' beaches, and our hence the state's tourism industry.

The goal of this project was to improve water quality management decisions in the state by 1) optimizing a rapid qPCR-based method for enterococci in Hawai'i, and 2) implementing a new qPCR-based method for human-associated *Bacteroides* (HBAC) as a sewage tracer to use in parallel with the EPA recommended enterococcus tests. If the HBAC assay can be validated as an adjunct to the enterococcus method it could be a way of generating faster results. This could help to improve the accuracy of and speed up beach management decisions (whether to post beach advisories).

Based on analyses of pure fecal samples collected from humans and several animals in Hawai'i, this project identified that the human specificity of HBAC HF183 is 74% and sensitivity 100%. The HBAC HF183 marker was detected in all untreated wastewater samples we collected at concentrations which exceeded enterococci's by approximately four orders of magnitude, indicating that it should be easily detected when sewage-borne enterococci are present. Correlation coefficient and Index of Agreement determined between the traditional cultivation-based method and the modified, more rapid, molecular method for enterococci examined by this project, met EPA's requirements for alternative methods (R^2=0.76 and IA=0.78) regarding how closely they matched the approved method. During our study, samples were routinely collected, analyzed and results reported within three hours. By implementing HBAC as a sewage tracer and by optimizing the enterococcus molecular-based method, more accurate and faster recreational water quality assessment will be possible. It is anticipated that, if adopted by the state the molecular method will positively affect reduce the rate of sewage-borne illness and related costs.

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CHAPTER 1

INTRODUCTION

1.1 Recreational Water Quality Criteria and Hawai'i

It was not until the 1900s that recreational water quality standards and sewage treatment requirements were introduced. The Federal Water Pollution Control Act (FWPCA) was introduced in 1948 as the first major United States law to address water pollution (USEPA, 2017). In 1970, the United States Environmental Protection Agency (USEPA) was created due to the rising concern in environmental pollution (Lewis, 1985). FWPCA has been amended a number of times since inception. In 1972, major amendments were made to FWPCA (USEPA, 2017). In 1986, Ambient Water Quality Criteria for Marine and Recreational Waters were release by the USEPA (USEPA, 1986). These water quality criteria were developed based on epidemiological studies conducted in coastal areas in the Northeastern US and the Great Lakes, and aimed at establishing a relationship between illness incidence and concentrations of fecal indicator bacteria (FIB) (Dufour, 1994).

FIB, such as enterococci and *Escherichia coli (E. coli)*, are typically not pathogenic, but when detected in quantities above water quality standards theoretically pose a threat to human health. Based on epidemiological studies, USEPA recommended using enterococci as the indicator of marine beach water quality, and *E. coli* or enterococci as the indicator of freshwater quality (USEPA, 1986). The use of these same FIB was also recommended by the latest version of the criteria (USEPA, 2012; Fujioka et al., 2015). Concentration of FIB in ocean waters has been utilized for decades to evaluate recreational water safety. However, there is still no agreement about which organisms (total coliforms, coliphage, fecal coliforms, *E. coli*, enterococci and others) are best suited as fecal indicators (Noble et al., 2003, Dufour, 1994, Fujioka et al., 2015).

USEPA's Recreational Water Quality Criteria (USEPA, 2012) serves as a guideline on which states develop and adopt their water quality standards. Hence, Hawai'i's water quality standards are based on these federal criteria, and use enterococci as an indicator of sewage contamination in marine as well as fresh recreational waters. These standards (DOH, 2014) require that microbiological parameters in recreational waters shall meet the following:

(a) *Enterococcus* shall be expressed in colony forming units (CFU) per 100 milliliters (mL) (100 ml^{-1}) or as most probable number (MPN) 100 ml^{-1}.

(b) *Enterococcus* content shall not exceed a geometric mean of 35 CFU or MPN 100 ml^{-1}over any thirty-day interval.

(c) A Statistical Threshold Value (STV) of 130 CFU or MPN 100 ml^{-1} shall be used for *Enterococcus*. The STV shall not be exceeded by more than ten percent of samples taken within the same thirty-day interval in which the geometric mean is calculated.

(d) Raw or inadequately treated sewage or other pollutants of public health significance, as determined by the director of health, shall not be present in natural public swimming, bathing or wading areas.

Currently beach advisories in Hawai'i are posted online or as physical signs at the beach

when enterococci concentrations exceed 130 CFU 100 ml^{-1}. Signs are only posted when the enterococci levels become chronically high ("Caution sign") or concentrations of *Clostridium perfringens* exceed 50 CFU 100 ml^{-1} ("Warning sign"). This latter criterion is frequently associated with known sewage spills (DOH, 2017).

Enterococcus, is one of the most studied microbes and research into it has increased over the years (Moreno et al., 2006). This genus is found in the gut of different animals but can survive and replicate in extra-enteric environments (Weber and Rutala, 1997; Deibel, 1964; Bale et al., 1993; Davies-Colley et al., 1994; Figdor et al., 2003). Enterococci can tolerate extreme pH, temperature, salts and detergent conditions (Huycke, 2002). Incubation is ideally conducted at an elevated temperature (42–45°C) (Reuter, 1992). Enterococci doubling time is 65 minutes (Figdor et al., 2003).

While sewage can be the origin of enterococci, several other sources also exist. Soils, sediments, bathers, aquatic vegetation, beach sand, and many other non-point sources may all contribute significant numbers of enterococci to water bodies (Viau et al., 2011; Byappanahalli et al., 2012, Cui et al., 2013). This can lead to unnecessary beach notifications, falsely alarming residents. In Hawai'i, background levels of enterococci in pristine waters naturally exceed the state standards by ten-fold or more, as enterococci can thrive in nutrient rich, moist and warm tropical environments (Hardina and Fujioka, 1991, Fujioka et al., 2015, Fujioka and Byappahalli, 2003) (Figure 1). While growth of enterococci in soils and their runoff into water bodies is a major contributor to water quality exceedances in Hawai'i, other sources may also contribute. For example, pig and mongoose (very common feral animals in Hawaii) feces contain high concentrations of enterococci. Therefore, these two animals are strong candidates for enterococci sources in Hawai'i (Fujioka et al., 2015; Oshiro and Fujioka, 1995). Other wild native and introduced animals common in Hawai'i, such as rats, chickens, pigs, dogs, cows, and goats, are also important sources of enterococci in Hawaiian waters (Strauch, 2011; Strauch et al., 2014).

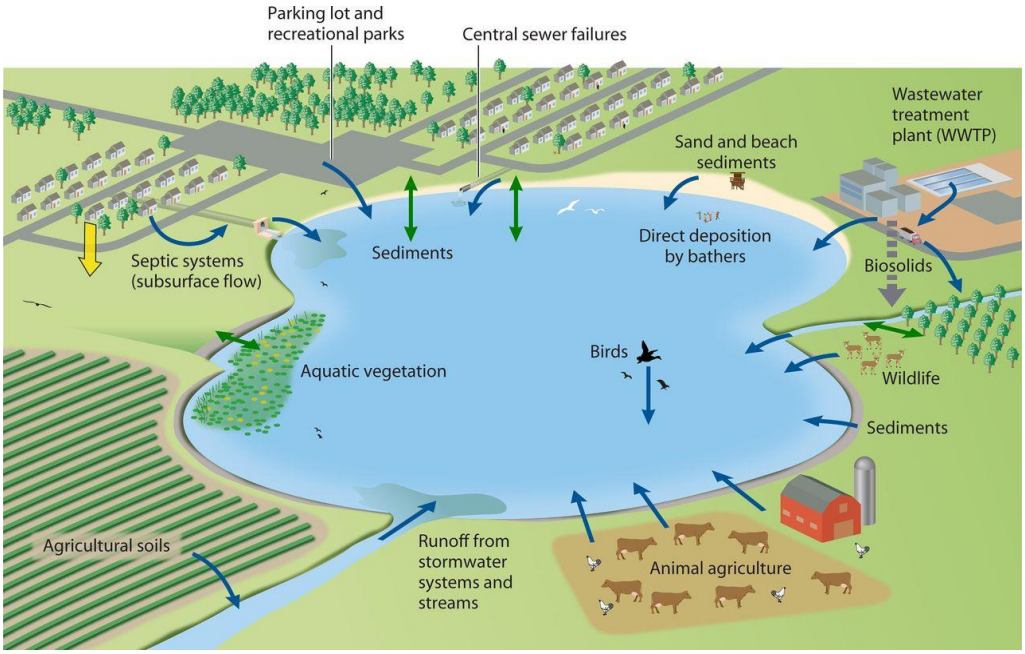

Figure 1: Sources of enterococci indicated by arrows. Sediment in water bodies contains reservoirs of enterococci, which can be re-suspended when the sediment is disturbed.

(Byappanahalli et al., 2012).

1.2 Microbial Source Tracking (MST)

Because environmental (non-sewage) sources of enterococci exist in Hawai'i, there is a need for methods to identify whether the enterococci are from sewage or from the environment. MST refers to a set of microbiological and chemical methods aimed at determining the source of microbial contaminants (Scott et al., 2002). The molecular marker-based approach is currently a promising MST method (Field et al., 2003). The idea behind this approach is that target organisms specific to different animal (humans, dogs, etc.) hosts exist, and these can be detected using molecular tools. The application of molecular markers can be hampered by low concentrations present in feces of target animals and/or cross-reactivity of markers from non-target species. The prevalence and specificity of any given marker is known to vary regionally, hence the markers need to be tested for sensitivity (% of individuals from the target species that have the marker) and specificity (% of individuals from non-target species that do not exhibit cross—reactivity with the marker (true negatives)) when applied at different locations (Layton et al., 2010; Stoeckel and Harwood, 2007; Harwood et al., 2014). Therefore, before markers developed elsewhere can be utilized in any location, they need to be evaluated based on the local animal and human population.

A sequence in the 16S RNA gene of *Bacteroides*, HF183 marker, has been suggested as an indicator of human sewage (Bernhard and Field, 2000) and has been used frequently in MST studies conducted in the mainland United States (Boehm, et al., 2013). Recent studies have suggested that the HF183 marker based assay is well suited for use in Hawai'i as concentrations are high in raw sewage and human feces in Hawaii, exceeding those of enterococci (Kirs et al., 2016). Also, as *Bacteroides* is an obligate anaerobe it cannot replicate outside a host (Bernhard and Field, 2000; Haugland, 2010). Application of this human-associated marker assay in parallel with tests for enterococci, would provide a higher degree of certainty about the presence of sewage when high concentrations of enterococci are detected, leading to fewer false positives and beach closures.

1.3 Methods for fecal indictor bacteria and MST markers

1. Cultivation-based methods for FIB.

Several different techniques to measure FIB concentrations in water samples exist. One of the most traditional techniques consists of filtering samples through membranes followed by incubation of the filters on FIB-specific media, such as mE or mEI medium for enterococci (USEPA, 2002), mTEC for *E. coli* (USEPA, 2002) and others. Other methods are multiple-tube fermentation (MTF) assays, which are well suited for turbid samples; and Enterolert®, basically a modification of the MTF in a tray format that uses multiple dilutions and a fluorogenic substrate that indicates the presence of the target bacteria (Budnick et al., 1996). These methods are easily learned and not costly. However, cultivation-based assays take about 18-48 hours to complete (Lavender and Kinzelman, 2009). Therefore, recreational water users can be exposed to sewage-contamination and pathogens as they have delayed information on beach water quality when they arrive at a beach. Cultivation-based assays tell you what the quality of the water was yesterday.

2. Molecular methods for FIB and MST markers.

Polymerase Chain Reaction (PCR)-based methods represent newer and more effective way of analyzing water samples for FIB and MST markers. PCR methods detect specific DNA

or RNA sequences from FIB or the markers. PCR was developed in the early 1980s by American chemist Kary Mullis for which he received Nobel Prize for chemistry in 1993 (Walker, 2002). A vast improvement over earlier versions of PCR is so called real-time PCR, which allows nucleic acid amplification to be monitored in real time during the analysis. When appropriate standards and controls are used, concentrations of nucleic acids (targeted by a given test) in a sample can be determined and consequently the concentration of a given group of bacteria determined. The real-time PCR assay is hereafter referred to in this text as quantitative PCR (qPCR) (Figure A.1). Modern qPCR machines perform rapid temperature cycling so that the requisite 40 cycles can be completed in less than an hour (Bartlett and Stirling, 2003), hence results can be obtained sooner than by using cultivation-based methods (Boehm et al., 2009; Bourlat et al., 2013). Typically, 96 reactions can be monitored simultaneously and multiplexing up to five assays is possible (Wilhelm and Pingoud, 2003).

Today, qPCR is the method of choice for the quantification of nucleic acids from specific organisms (absolute or relative) and a wide variety of protocols, PCR chemistries and instruments are utilized (Bustin, 2005). The qPCR method consists of comparing the fluorescence from each reaction vessels, typically a strip of tubes or a plate containing wells, (Figure A.2) to a pre-determined standard. During each of the 40 amplification cycles (C_t), the DNA or RNA is denatured, annealed and elongated, which produces fluorescence, which is measured by the qPCR's machine optical module (Wilhelm and Pingoud, 2003). The fluorescence signal is then converted into a curve, which shows fluorescence intensity on the y-axis and the number of amplification cycles on the x-axis (Figure A. 3). The curve also provides us with efficiency (slope of the standard curve) and correlation coefficient (R^2) values. Efficiency indicates how effectively DNA is amplified during each cycle (100% indicates doubling of target DNA in each cycle). R^2 measures the correlation between the counts determined in the standards compared to those for the samples. Optimal values for in assays are between 90% and 110% for efficiency and 0.990 for R^2.

QPCR reactions typically contain DNA or RNA of interest, water, two primers (single-stranded target specific oligonucleotides), a probe, nucleotides (A, T, C, G), and DNA polymerase all mixed in a buffer (Bartlett and Stirling, 2003). Probes are oligonucleotides that are labeled with a fluorescent reporter and a quencher. The quencher decreases fluorescence intensity (Wilhelm and Pingoud, 2003). During each PCR cycle the probe denatures and anneals to the target sequence. For every amplification of the target sequence a fluorescent reporter is released from the probe. The qPCR has two main components: a thermal cycler and an optical module (to detect fluorescence in the reactions during the thermal cycling). The thermal cycler performs three major steps by cycling the temperature: denaturation, annealing and elongation of target DNA (Wilhelm and Pingoud, 2003). Optimally during each cycle, specific target DNA should double and the fluorescence in each reaction in the plate increases as the probe, which fluoresces the sample, is released due to the exonuclease activity of the DNA polymerase. The increase in fluorescence is converted into an amplification plot/curve (Figure A. 3). The baseline of the amplification curve represents the number of cycles where there is a little change in fluorescence signal. The threshold is the level of signal that reflects a statistically significant increase over the calculated baseline signal (Wilhelm and Pingoud, 2003). C_t value is the cycle number where the fluorescence increases above baseline (crosses the threshold line) (Wilhelm and Pingoud, 2003). The amplification curve has an exponential, linear and plateau phase. The exponential phase is when the sequence is doubling at every cycle. The linear phase is when the reaction components are being consumed, which causes the reaction to slow down as products

degrade. The plateau phase is reached when the amplification of nucleic acids is no longer possible as the ingredients have been depleted (Wilhelm and Pingoud, 2003).

QPCR has great potential in water quality monitoring programs where it can be used to detect different microorganisms, including human pathogens and MST markers. Furthermore, as the quantification process is fast, it can be used for rapid water quality testing, so that water quality warnings can be posted the same day samples are collected, preferably before most people arrive at a beach (Noble and Weisberg, 2005). Two qPCR-based methods (method 1609 and 1611) have been approved by EPA for states to use in their water quality monitoring programs for beach notification purposes (USEPA, 2012). Studies utilizing these methods or their derivatives have shown that there is good agreement between the concentrations of enterococci determined by cultivation-based and qPCR-based methods (Gonzalez and Noble, 2014, Haugland et al., 2016). Furthermore, in recent epidemiological studies conducted in Mississippi, Rhode Island and Alabama in 2005-2007, there was a strong association between the qPCR derived enterococci measurements and occurrence of gastrointestinal illnesses observed (adjusted odds ratio (AOR) = 2.6, 95% CI 1.3-5.1, where AOR=1 would indicate no association), although it needs to be emphasized that these studies was conducted at a sewage-impacted beach where most of the enterococci were known to be of sewage origin (Wade, et al 2010).

While several states have already started implementing qPCR tests in their water quality monitoring programs, Hawai'i is trailing in the adoption of the method. The adoption of this method for beach notification purposes in Hawaii might reduce public exposure to potentially contaminated water, because it is more rapid, hence reports more timely results to the public. Moreover, there are several potential economic benefits as the incidence of water borne illness diseases might be expected to decrease. Also, beaches can be deemed safe to swim sooner after contamination has receded.

1.4 Wastewater treatment and Hawai'i

Municipal wastewater contains many pollutants such as organic matter, nutrients, chemicals, suspended solids, and pathogenic organisms. These components of wastewater can negatively impact receiving waters. For example, chronic discharge of human sewage into the environment without treatment can result in eutrophication and decreases in biodiversity (Smith et al., 1999). When sewage is introduced into aquatic environments where there are limiting nutrients, it can result in plant overgrowth and algal blooms. When the blooms die, they deplete large amounts of oxygen from the water, causing the aquatic environment to become anoxic. The reduction of dissolved oxygen leads to changes in ecosystem structure and function, often suffocating aquatic life (Smith et al., 1999). Eutrophication can also affect fish, birds and entire food chains (Sharpley et al., 1994). In Hawai'i, the situation is somewhat different. Facilities such as Sand Island Wastewater Treatment Plant (WWTP) discharges its effluent 1.7 miles offshore and in 230 feet of water. Onshore plants such as Waimanalo WWTP injects its treated effluent into injection wells (Department of Environmental Services, 2017). In either case, the effluent is quickly and massively diluted, reducing the chances of causing eutrophication to a minimum.

Likewise, human life is negatively impacted if untreated sewage discharge impairs ecosystem services such as swimming, drinking and fishing. More importantly, water contaminated with human feces poses a direct health risk (Wade et al., 2010) and untreated sewage represents the greatest health risk as it usually contains a wide variety of human

pathogens, while animal waste does not (Fujioka et al., 2015). A recent study (Arnold et al., 2016) suggested that exposure to contaminated water represents the most important cause of acute gastroenteritis among American children. In summary, contamination of recreational waters by untreated or partially treated sewage can negatively impact the environment as well as lead to the spread of several diseases, which may result in an increase the incidence of disease and mortality (Wade, et al 2010).

Wastewater treatment technology has been developed to provide adequate treatment of sewage prior to releasing it into receiving waters (Mallin et al., 2007). Wastewater treatment processes can be classified into three categories: physical (screening, sedimentation, filtration), chemical (flocculation, chlorination, ozonation) and biological (activated sludge, anaerobic digestion, trickling filters). These processes can be performed in combination or separately (Olsson and Newell, 1999). Wastewater is thus categorized into three types based on the degree of treatment it has undergone. Primary influent is the raw sewage arriving the WWTP. Primary effluent is wastewater has gone through a first cleaning stage (physical treatment) that typically removes 30-40% of organic matter and pathogens (Menegaki et al., 2007). Secondary effluent is primary effluent that has gone through a process to remove more BOD, suspended solids and nutrients, such as nitrogen and phosphorus that could cause eutrophication (Jiménez et al., 2010). After the secondary treatment, the organic load should be reduced by 95% (Menegaki et al., 2007). The next level of wastewater treatment (tertiary), which usually includes disinfection (UV, ozonation and chlorination), yields water that ideally does not contain pathogens and a 99% reduced organic load.

The island of Oahu has ten different WWTPs and together they serve most of the island's population (82%) (Figure 2) (Table A. 1) (Houghton, 2012). After processing through these WWTPs, the discharged water should not compromise the quality of recreational waters, where bacterial levels should meet Hawai'i's recreational water quality standards (Mallin et al., 2007).

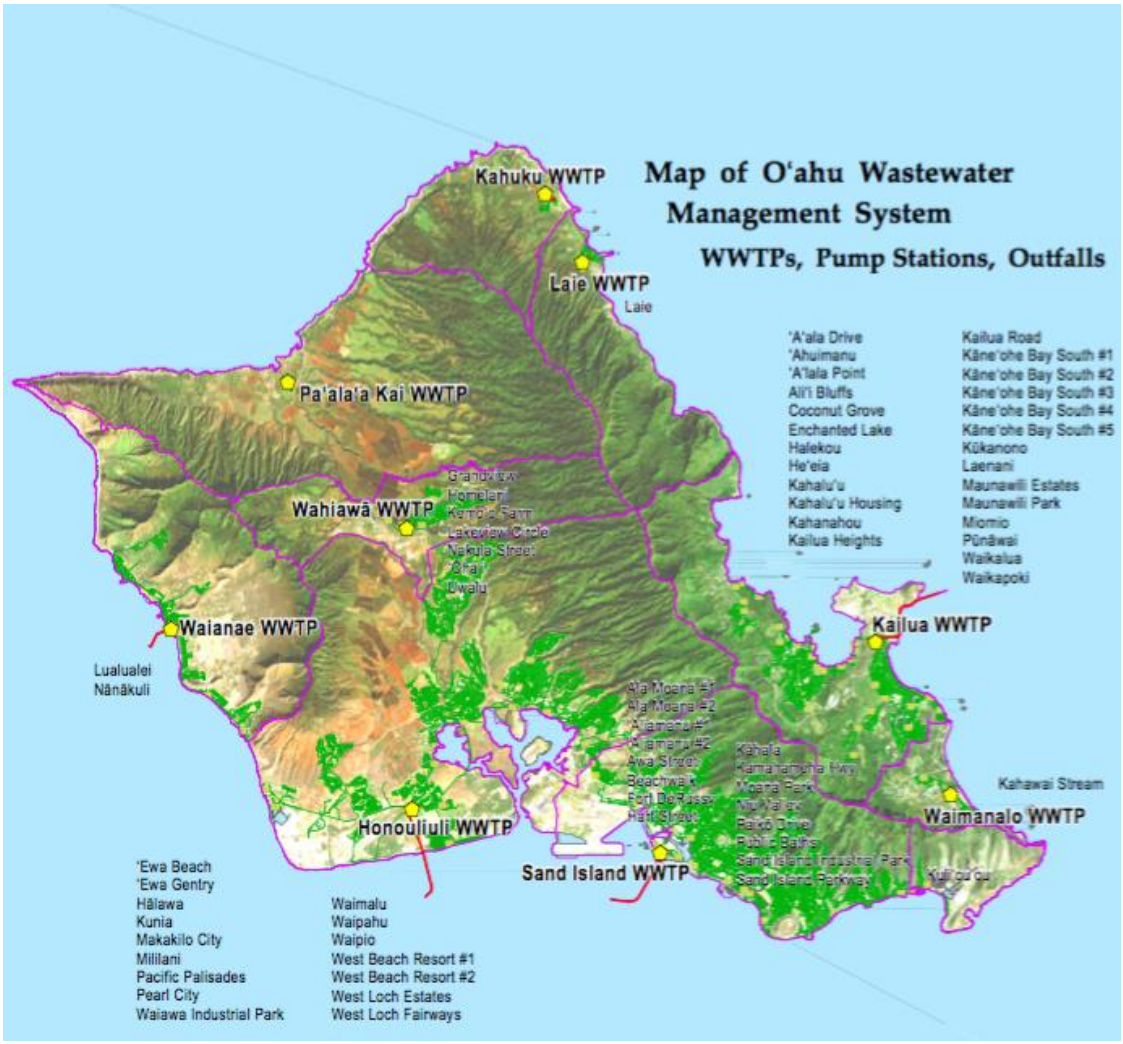

Figure 2: Locations of WWTPs on O'ahu (Department of Environmental Services, 2017).

Previous studies have identified that in 2001 alone there were over 40,000 sewer overflows and 400,000 backups of raw sewage into basements in the U.S. (Dorfman et al., 2004). Hawaii often deals with sewer overflows, many of our sewer lines are approaching 100 years of age and inflow resulting from heavy rains causes the system to frequently overflow on Oahu (Fletcher et al., 2010). In the 1990's Oahu's system had 200 spills, overflows and bypasses mostly caused by heavy rains and breaks in sewer lines (Boylan, 2008; Fletcher et al., 2010). These spills represent significant sources of human sewage pollution to the environment, hence reliable methods to detect sewage-borne microbial contamination and estimate related health risks are needed.

CHAPTER 2

PROJECT OVERVIEW AND OBJECTIVE

Today, the Hawai'i Department of Health (HIDOH) and the City and County of Honolulu (CCH) use culture-based methods for enterococci and *Clostridium perfringens* to evaluate beach water quality (Fujioka et al., 2015; DOH 2017). These methods are slower than qPCR (24-48 h compared to 2-3 h) and do not provide near real-time information to the public. Hence, qPCR-based methods, which have been approved for the states to use for rapid beach notification purposes by the latest federal recreational water quality criteria (USEPA, 2012), may reduce the risk to beach goers by providing near real-time water quality information.

Before these methods can be utilized in water quality monitoring programs in Hawai'i, they need to be evaluated under local conditions. When the methods were published in 2012-2013, only two samples from Hawai'i had been tested by the EPA (Kirs M., personal communication). An earlier study conducted in Hawai'i by the University of Hawaii's Water Resources Research Center laboratory demonstrated that a large proportion of samples (~70%) collected in Hawai'i could not be analyzed using rapid methods due to losses of DNA during the rapid DNA extraction process specified by both EPA protocols (Kirs, et al., 2017). Adjustment of sample pH to 3.5 prior to filtration removes suspended calcareous (coralline) particles and appears to alleviate the issue.

The recommended chemistry for qPCR specified by the EPA protocols is suited for relatively slow temperature cycling, while modern PCR machines and chemicals allow protocols which utilize more rapid cycling of temperature which can accelerate the entire process and allow more timely dissemination of results.

This project tested an alternative PCR chemistry to achieve more rapid results. QPCR reactions using the new chemistry can be completed faster (within 64 min) compared to EPA's recommended chemistry (> 1hour and 40 minutes). EPA has provided guidelines for validating alternative methods, which are found in their Site Specific Alternative Recreational Criteria (SSA) (USEPA, 2014). Alternative proposed methods must be compared to the EPA-approved method and they must agree based on the correlation coefficient (R^2>0.6) and Index of Agreement (IA≥0.7), which is a measurement of the degree of model predication error (ranges from 0-1) (Willmott, 1981) (USEPA, 2014). As we modified the EPA-approved protocol (acidification and rapid PCR chemistry), we needed to test our protocol and compare the results to those obtained using the approved protocol. We tested whether the modified protocol met the EPA guidelines.

As discussed in the introductory section, enterococci are excreted by many animals and can naturalize and grow in extra-enteric environments such as soils, sediments and beach sand (Hardina & Fujioka, 1994; Byappanhalli & Fujioka, 2003; Kirs et al., 2016; Cui et al., 2013). Therefore, *Enterococcus* is not an accurate indicator of human sewage in Hawai'i and an alternative tracer to confirm sewage contamination is needed. HBAC is an ideal candidate as an alternative sewage-specific indicator, as it cannot grow in the presence of oxygen (most environments outside the host), and its concentrations in sewage typically exceed concentrations of enterococci. Several assays for HBAC already exist (Boehm et al., 2013). A multi-laboratory study (n=21) of 41 MST methods concluded that the HBAC HF183 endpoint assay and the HF183TaqMan (Haugland, 2010) assays are the most sensitive and specific (Boehm et al., 2013). The HF183TaqMan assay has been further modified and improved by Green et al. (2014). This version of the assay is hereafter referred to as the BacHF183/R287 assay and it was used in this

study to determine concentrations of HBAC.

This improved version of HF183TaqMan marker has not yet been validated in Hawai'i so it was important to validate this marker using local fecal and sewage samples, because animal populations, human diets and related gut flora vary from place to place, as do water temperature, sunlight and decay rates (Kirs et al., 2016). Research has shown that in Hawai'i concentrations of *Bacteroides* quantified using the earlier version of HF183TaqMan as used in Haugland et al. (2010) exceeded concentrations of enterococci in a 1,355:1 ratio in human fecal samples, indicating that this marker can be a more sensitive indicator for sewage contamination (Kirs et al., 2016). Even though *Bacteroides* is present in all wastewater samples and most human fecal samples, in earlier studies the HF183TaqMan protocol detected it in dog, cat and mongoose samples. Hence, the HF183TaqMan assay does not have high specificity in Hawai'i (74%) (Kirs et al., 2016). Recent studies have shown that the modified TaqMan assay (BacHF183/R287) alleviates nonspecific amplification problems (Green et al., 2014), hence possibly improving specificity and the limit of detection of the assay. Furthermore, as sewage and the enterococci in it are rapidly diluted in the environment, it is important that concentrations of any proposed sewage-specific tracer should be similar or exceed concentrations of enterococci in raw sewage. It is hypothesized that concentrations of HBAC (as quantified using BacHF183/R287) exceed or are similar to concentrations of enterococci in sewage. This would enhance detection of sewage in the environment if the marker is truly sewage-specific. In order to evaluate the specificity and sensitivity of the modified assay, we collected and analyzed primary influent, primary effluent, secondary effluent and tertiary effluent from Honouliuli WWTP, Sand Island WWTP and Hawaii Kai WWTP. The three WWTPs were chosen because they process different volumes of flow and serve three different areas of the island, therefore together they are representative of the gut fauna of Oahu's inhabitants. Six different animal fecal samples were also examined (see project description). To evaluate the feasibility of the method a three-week beach monitoring was conducted. This was followed by two weeks of evaluation of water from the Ala Wai Canal (a tidally influenced drainage canal near Oahu's south shore) since the beach samples we obtained contained very low numbers of enterococci, making the comparison of the two methods difficult.

The overarching goal of this project was to improve Oahu's water quality management decisions by 1) optimizing EPA's rapid qPCR method 1611 for enterococci in Hawai'i, and 2) implementing a new qPCR-based method for HBAC as a sewage tracer in parallel with the EPA recommended *Enterococcus* tests. To evaluate and implement the new rapid water quality methods in Hawai'i, the following tasks were executed:

- Task 1: Setup of qPCR assays for enterococci (USEPA 1611, 2012) and HBAC (BacHF183/R287TaqMan (Green et al., 2014)) to determine efficiency of amplification reactions as well as limits of quantification and detection based on the standards used.
- Task 2: Validation of the BacHF183/R287TaqMan assay for specificity and sensitivity using animal and human fecal samples, as well as wastewater samples.
- Task 3: Comparison of enterococci and HBAC concentrations in sewage.
- Task 4: Analysis of coastal water quality (Ala Moana Bowls, Kahanamoku Beach and Lagoon) (Figure A. 4) using cultivation-based and modified molecular *Enterococcus* assays (methods 1611, and Enterolert®) to evaluate the utility of method implementation (i.e. can the method provide timely results for beach

notification?).

- Task 5: Analysis of water quality in Ala Wai Canal (Figure A. 5) (brackish water) using cultivation-based (Enterolert®) and a modified molecular *Enterococcus* assay (modified version of method 1611). Based on task 4 and 5 evaluate whether the modified, more rapid, version of the test will pass EPA guidelines for alternative recreational water quality methods.

CHAPTER 3

MATERIALS AND METHODS

3.1 Procedure for Task 1: Setup of the qPCR assays for enterococci and HBAC to determine if our laboratory procedures are working properly (

Table *1*).

Table 1: qPCR primers and plasmid target constructs.

Assay	Forward primer	Reverse primer	Probe	Reference
Sketa	5′-GGTTTCCGCAGCTGGG	5′-CCGAGCCGTCCTGGTC	5′-FAM-AGTCGCAGGCGGCCACCGT-BHQ	(Haugland et al., 2012)
HBAC	5′-ATCATGAGTTCACATGTCCG	5′-CTTCCTCTCAGAACCCCTATCC	5′-FAM-CTAATGGAACGCATCCC-MGB	(Green et al., 2014)
Enterococcus	5'-GAGAAATTCCAAACGAACTTG	5'-CAGTGCTCTACCTCCATCATT	5'-TGGTTCTCTCCGAAATAGCTTTAGGGCTA-TAMRA	(Haugland et al., 2012)

A. Hypothesis

Hypothesis 1.1. The qPCR for enterococci and BacHF183/R287TaqMan will meet the criteria set forth in the expected results section of the relevant MIQE and EPA guidelines (MIQE, 2009, USEPA, 2013).

B. Experiments to test hypothesis 1.1

Experiment 1.1. Run serially diluted standards for enterococci assay in triplicate to identify the lower limit of detection (LLD) (minimum concentration at which marker is detected) and quantification (LLQ) (minimum concentration at which marker is quantified), range of detection (linear dynamic range), R^2 (correlation between observed and expected concentration based on the standards) and efficiency of amplification, for modified qPCR test. Purified cells (see below) were used as standards at concentrations 5, 50, 500 and 5,000 target copies per reaction.

Experiment 1.2. Run serially diluted standards for BacHF183/R287TaqMan assay to identify LLD and LLQ, range of detection (linear dynamic range), R^2 and efficiency of the amplification, for modified qPCR test. Linearized purified plasmids (see below) were used as standards at concentrations 0.83, 8.3, 83, 830, 8,300, 83,000, 830,000 target copies per reaction. Concentrations <100 target copies were tested in triplicate while concentrations >100 target

copies were tested in sextuplicate reactions.

Filtration (USEPA 1609, 2013)

Quantification standards:

*Enterococcus faecalis (*ATCC® #29212) cell suspension was removed from -80°C storage and thawed at room temperature. Cells were streaked onto brain heart infusion agar and grown for 24 hours at 37°C. A single colony was isolated from the agar plates and incubated in 10 ml of brain heart infusion broth for 24 hours at 37°C. The turbid tube with cells was centrifuged for five minutes at 6,000X g to collect cells. Cells were washed in phosphate buffered saline three times using the same centrifugation speed. Final cell concentrations were determined by serial dilution and plating on brain heart infusion agar (Option 2 in the USEPA Method 1611). Cells were aliquoted and stored at -80°C as single use standards.

The linearized plasmid standard for the BacHF183/R287TaqMan assay was prepared as follows: firstly, the target DNA sequence was synthesized and inserted into a plasmid by Integrated DNA Technologies (Integrated DNA Technologies, Coralville, IO). Secondly, we linearized and purified this plasmid. Thirdly, we quantified the plasmid using dsDNA HS Assay Kit on a Qubit® 2.0 fluorometer (Life Technologies; Carlsbad, CA). The linearized and quantified plasmid was aliquoted and stored at -80°C as single use standards.

Ten µl of reference material (*E. faecalis* cells or BacHF183/R287TaqMan plasmid) were each spiked into AE Buffer solution per EPA guidelines (Qiagencat # 19077) containing 0.30 ± 0.03 g acid-washed glass beads (Sigma-ALDRICH Co., St. Louis, MO) and run through a rapid DNA extraction protocol which included bead beating in a mini beadbeater (Mini-BeadBeater-8, Biospec Products Inc. Bartlesville, OK) to extract the DNA into solution. This was followed by centrifugation for one minute at 12,000X g. The supernatant was transferred into a 1.7 ml microtube and centrifuged again for five minutes at 12,000X g. After this the supernatant was removed, and serially diluted in AE buffer and used as template in subsequent qPCR tests to determine the LLD and LLQ of both assays.

QPCR:

QPCR was performed in a CFX96™Real-Time System (Bio-Rad Laboratories, Inc, Hercules, CA) using TaqMan®Environmental PCR master mix 2.0 (Bio-Rad Laboratories, Hercules, CA) optimized for the system and rapid temperature cycling instead of USEPA recommended Environmix 2.0 (ThermoFisher Scientific, Waltham, MA). The reaction in each well contained TaqMan®Environmental PCR master mix 2.0 (Bio-Rad Laboratories, Hercules, CA), 0.2 mg/ml bovine serum albumin (New England Bio Lab, Ipswich, MA), 1 µM of each primer, 80 nM FAM™labeled TaqMan®probe (Life Technologies, Carlsbad, CA) and plasmid DNA extracts in a total reaction volume of 25 µL. The primer and probe sequences were assay specific. All reactions were performed in triplicate in AxygenPCR® white 96-well reaction plates (Axygen. INC, Union City, CA) with MicroAmp Optical Caps (Applied Biosystems). The plates were placed in a balanced plate spinner and spun for ten seconds. Thermal cycling conditions were ten minutes at 95°C (enzyme activation), followed by 40 cycles between fifteen seconds at 95°C (DNA denaturation) and two minutes at 60°C (DNA annealing and elongation). Data was analyzed using Bio-Rad CFX Manager software (Version 3.1). Threshold cycle (C_T) values were exported to Microsoft Excel for further statistical analysis. Samples containing PCR inhibitors

were determined by challenging PCR reactions containing equal concentrations of salmon testes DNA (Sketa control DNA) (Sigma-Aldrich; St. Louis, MO) with 5 µl of an unknown sample as in Haugland, et al., (2005) (Sketa assay). Samples for which the threshold cycle was delayed by more than 3.3 PCR cycles compared to reactions challenged by molecular grade water (ΔC_t) were considered inhibited and subjected to a ten-fold dilution with molecular grade water to dilute the inhibitors.

C. Expected Results

The following results were expected after task I based on guidelines (MIQE, 2009, USEPA, 2013):

- Detection range of four to six orders of magnitude (5, 50, 500 and 5,000 and 0.83, 8.3, 83, 830, 8,300, 83,000, 830,000).
- Standard's R^2>0.97
- Efficiency: 90-110%
- LLQ 20 copies per reaction (which corresponds to 590 cell equivalents in 100 ml)
- LLD- five copies per reaction, which corresponds to 147 cell-equivalents and >95%

3.2 Procedure for Task 2: Validation of the BacHF183/R287TaqMan assay for specificity and sensitivity using animal and human fecal samples, as well as wastewater samples.

A. Hypotheses

Hypothesis 2.1. The assay that we tested will be 100 percent specific to its target (humans). This means that the HBAC detected originates only from human feces and sewage samples and no cross-reactivity will be detected with feces from other animals.

Hypothesis 2.2. The assay we tested will be 100 percent sensitive to its target (humans). This means that HBAC is shed by all humans.

B. Experiments to test hypotheses 2.1 and 2.2

Experiment 2.1. To determine sensitivity and specificity of the BacHF183/R287TaqMan assay, an array of human and animal fecal samples were analyzed for the marker using qPCR.

Fecal Samples:

DNA from fecal samples collected by Kirs et al (2016) from various animals and humans were available for this study. Single stool samples were provided by ten human volunteers ages 33 to 59 (1:1 female to male ratio) between 04/24/2013 and 06/24/2013. These samples were collected according to the protocol approved by the Institutional Review Board of the University of Hawai'i (Notice CHS 21096; Human Studies Program, University of Hawai'i) using aseptic stool specimen collection kits. Fecal samples originating from ten individual cats (*Felis catus*) were collected across the University of Hawai'i Mānoa campus in Honolulu, between 03/25/2013 and 03/22/2014. Fecal samples from ten individual rats (*Rattus rattus*) and ten individual mongooses (*Herpestes javanicus*) were collected between 03/05/2013 and 05/02/2013 on Hawai'i Island (Big Island). Eleven individual domestic dog (*Canis lupus ssp. familiaris*) samples were collected from various sites on the island of O'ahu (Aiea, Kaimuki, Kalihi, Kaneohe, Mānoa, Waipahu and downtown Honolulu) and five chicken (*Gallus gallus*) samples

were collected near the University of Hawai'i at Mānoa lower campus in May 2015. Ten feral pig (*Sus scrofa*) samples were collected 10/30/ 2015 in a forest adjacent to the laboratory of the HIDOH at Waimano Home Road on Oahu.

qPCR**:**

QPCR was performed as optimized in task 1. Three µl of fecal DNA were analyzed in duplicate, while five-point standards were evaluated in triplicate.

C. Expected Results

BacHF183/R287TaqMan is expected to be detected in human samples only.
BacHF183/R287TaqMan is not expected to be present in animal samples.
This would give the marker 100% specificity
BacHF183/R287TaqMan is expected to be detected in all human samples.
This would give the marker 100% sensitivity

3.3 Procedure for Task 3: Comparison of enterococci and HBAC concentrations in sewage.

A. Hypothesis

Hypothesis 3.1. The BacHF183/R287TaqMan marker will be present at high concentrations in Hawai'i wastewater samples, exceeding concentrations of enterococci.

B. Experiments to test hypothesis 3.1

Experiment 3.1. Analysis of wastewater samples for enterococci and BacHF183/R287TaqMan marker concentrations.

Wastewater Samples:

These samples were used to determine the concentrations of the two molecular markers (enterococci and *Bacteroides* using the BacHF183/R287TaqMan assay) compared to cultivable enterococci concentrations in raw sewage. Wastewater samples were collected using 1-L sterile bottles at three treatment plants (SIWWTP, HOWWTP, HKWWTP) between February and March 2017. At the SIWWTP samples were collected from primary influent/effluent and after UV disinfection. At the HOWWTP samples were collected from primary influent/effluent, secondary effluent and R1. HKWWTP samples were collected from primary influent/effluent, secondary effluent and after UV disinfection. All wastewater samples were transported to the laboratory in a cooler on ice and analyzed within three hours. In the laboratory samples were serially diluted with deionized (DI) water and cultivable enterococci concentrations were determined using the Enterolert® system in Quanti-Tray®/2000 format (IDEXX Laboratories, Inc.; Westbrook, ME). DNA for the molecular tests was extracted using a membrane filtration method. Briefly, 100 mL of disinfected and/or R1 samples, 50 mL of primary and UV treated, and secondary effluent samples, 20 mL of primary effluent samples, and 10 mL of primary influent samples were filtered through a Nucleopore Track-Etch Membrane Filter (47 mm diameter, 0.4 µm pore size; Whatman, Maidstone, UK). As a negative control, 100 mL of sterile Milli-Q®Integral Water was filtered (Millipore, Billerica, MA). Following the filtration, the membranes containing the bacterial cells and DNA were placed in 2.0 ml micro-centrifuge tubes with 0.30 ± 0.03 g acid washed glass beads (Sigma-ALDRICH Co., St. Louis, MO), 600 µL of buffer (588 µL of AE Buffer (Qiagencat# 19077) and 12 µL of 0.01 µg/µL Sketa control DNA

(Sigma-ALDRICH Co., St. Louis, MO) was added to the tubes, and then shaken at high speed in a mini beadbeater to extract the DNA into solution. The supernatant was centrifuged (one minute, 12,000X g), transferred into a 1.7 ml microtube and centrifuged again (five minutes, 12,000X g). Standards were extracted as in Task1.

QPCR analyses for enterococci and BacHF183/R287TaqMan were performed as optimized in Task1.

Analyses of variance (ANOVA) was used to compare log-transformed concentrations of enterococci and HBAC in primary influent samples. Tukey test was used for pairwise multiple comparisons and the differences were considered significant when $p<0.05$.

C. Expected Results

Concentrations of BacHF183/R287TaqMan will exceed concentrations of enterococci in wastewater samples.

3.4 Procedure for Task 4: Analyses of coastal water quality using cultivation-based and modified molecular *Enterococcus* assays to evaluate the feasibility of using the new method for rapid beach notification.

A. Hypotheses

Hypothesis 4.1. When samples are collected at 6:00-7:00 am at Ala Moana Bowls, Kahanamoku Beach and Lagoon, results for both molecular tests can be posted by 10:00 am.

Hypothesis 4.2. There will be good correlation between the enterococci measurements obtained by molecular and Enterolert® cultivation techniques (IA≥0.7 or R^2>0.6 (USEPA, 2014)).

B. Experiments to test hypotheses 4.1 and 4.2

Experiment 4.1. Analyses of marine coastal samples collected daily over three-week period (6/26/17 to 7/14/17).

Ocean Water Samples:

Daily water samples were collected by Surfrider Foundation O'ahu Chapter (SFOC) and WRRC teams over a period of three weeks from 6/26/17 to 7/14/17 (n=15) (Table A.10) at Ala Moana Bowls, Kahanamoku Beach and Lagoon, O'ahu between 6:00 and 7:00 AM. These samples had their pH lowered to 3.5 using hydrochloric acid (HCl) (to remove the calcareous particulates) and were analyzed for concentrations of enterococci using rapid method 1611, modified by the use of SSOAdvanced chemistry (Bio-Rad Laboratories, Inc, Hercules, CA) as in Task 1. Concentrations of HBAC were determined using BacHF183/R287TaqMan as in Task 1. 100 ml water samples were used for both tests and extracted as in Task 3 for qPCR analyses. The same DNA extract was used for *Enterococcus* and HBAC tests. Concentrations of cultivable enterococci were determined using the Enterolert® system in Quanti-Tray®/2000 format (IDEXX Laboratories, Inc.; Westbrook, ME) before the samples were acidified. The performance of the two molecular methods modified 1611 (enterococci) and HBAC (*Bacteroides*) relative to the culture based method, time to report, *etc*., were evaluated. Every day during the testing period, the results were provided to the SFOC who reported the data to the public.

3.5 Procedure for Task 5: Analyses of water quality in Ala Wai Canal using cultivation-based and a modified molecular *Enterococcus* assays. Evaluate whether the modified test will pass EPA guidelines for alternative recreational water quality methods.

A. Hypothesis

Hypothesis 5.1. There will be good correlation between the enterococci measurements obtained by the cultivation and modified molecular technique (IA>0.7 or R^2>0.6 (USEPA, 2014)).

B. Experiments to test hypothesis 5.1

Experiment 5.1. Analyses of Ala Wai samples collected daily over a two-week period (7/17/17 to 7/29/17).

Ala Wai Water Samples:

Daily water samples were collected by SFOC and WRRC teams for a period of two weeks 7/17/17 to 7/29/17 (n=12) (Table A.10) at Ala Wai Canal between 6:00 and 7:00 am. Sample pH was lowered to 3.5 using hydrochloric acid (HCl) and samples were analyzed for concentrations of enterococci using cultivation and molecular tests as in section 4. Concentrations of HBAC were determined as in Task 4. The performance of the modified molecular *Enterococcus* test was compared to the cultivation-based test. HBAC marker analysis was done in parallel in order to evaluate the sources of contaminants in the samples collected.

Analyses of variance (ANOVA) was used to compare log-transformed concentrations of enterococci and HBAC in primary influent samples. Tukey test was used for pairwise multiple comparisons and the differences were considered significant when $p<0.05$.

CHAPTER 4

RESULTS

4.1 Task 1: Setup of the qPCR assays for enterococci and HBAC to determine if our laboratory procedures are working properly (

Table *1*).

Efficiency and R^2 for the enterococci standard were 98.2% and 0.996 (one test). Efficiency and R^2 for HBAC were 100.4 and 103%, 0.998 and 0.999 respectively (two tests).

LLQ for enterococci and HBAC was 20 cells 100 ml^{-1} and 20 gene copies (gc) 100 ml^{-1}respectively. The LLD for both assays were five cells or gc per 25 µl PCR reaction, which represents 100 cells or gc per sample (100 ml).

4.2 Task 2: Validate the HBAC assay for specificity and sensitivity.

4.2.1 Concentration of enterococci and HBAC in human and animal samples

Table 2: Concentrations of enterococci and HBAC in animal samples

Animal	Sample Size	Enterococci (cells/g) Average(min-max)	HBAC (gc/g) Average(min-max)	Sensitivity (%)	HBAC:enterococci ratio
Chicken	5	$2.92X10^7$ ($7.17X10^5$-$1.22X10^8$)	$5.12X10^4$ (<111-$1.05X10^5$)	40%	0.00175:1
Cat	8	$1.56X10^6$ ($9.12X10^3$-$1.07X10^7$)	ND (<111)	0%	N/A
Dog	11	$2.88X10^6$ ($2.97X10^4$-$2.62X10^7$)	$8.83X10^6$ (<111-$1.17X10^8$)	91%	3.1:1
Human	10	$1.20X10^6$ ($2.60X10^4$-$6.74X10^6$)	$1.92X10^9$ ($4.38X10^3$-$1.93X10^{10}$)	100%	1,840:1
Mongoose	10	$2.72X10^7$ ($1.82X10^5$-$2.06X10^8$)	$3.82X10^4$ (<111-$5.24X10^4$)	20%	0.0014:1
Pig	10	$1.77X10^6$ ($1.08X10^5$-$9.58X10^6$)	ND (<111)	0%	N/A
Rat	10	$2.23X10^6$ ($7.90X10^3$-$1.70X10^7$)	ND (<111)	0%	N/A

*Not detected

Concentrations of HBAC were higher than those of enterococci in all human samples, which suggests that HBAC is more sensitive than enterococci for detecting sewage contamination. The ratio of HBAC to enterococci was greater for human samples than for animal samples (

Table ***2***). The relatively high concentrations of HBAC in dog samples can be a concern since the marker was detected in ten out of eleven samples.

Concentrations of enterococci varied among animal samples. The highest average enterococci concentrations were found in chicken, cat and mongoose samples while the highest

concentrations of HBAC were found in human samples. Enterococci were detected in four out of five chicken samples, four out of eight cat samples, all of the eleven dog samples, eight out of ten pig samples, seven out of ten human samples, nine out of ten mongoose samples, and nine out of ten rat samples (

Table ***2***).

4.2.2 Sensitivity and specificity of HBAC in human and animal feces

HBAC was detected in all the human samples (100% sensitivity) and in all the wastewater samples (100% sensitivity) (*Table **3***,

Table ***2***). However, HBAC was also detected in animal samples. HBAC was detected in two out of five chicken samples (40% sensitivity), ten out of eleven dog samples (91% sensitivity), and two out of ten mongoose samples (20% sensitivity). No HBAC was detected in pig, rat or cat samples (

Table ***2***). The overall specificity of this marker was therefore 74%. Even though present in some animals, concentrations of HBAC were significantly higher in human samples compared to the animal samples. The average concentration ratio of HBAC found in human samples and animal samples (all non-human species) was: 251:1compared to dog, 43,329:1 compared to chicken, and 58,079 compared to mongoose. No comparison was possible for rat, cat and pig samples because the marker wasn't detected at all in samples from these animals.

4.3 Task 3: Compare concentrations of both assays in sewage

4.3.1 Cultivation-based concentrations of enterococci in sewage

In primary influent average enterococci concentrations were highest at the SIWWTP, followed by HKWWTP and HOWWTP. In the final effluent, average concentration of enterococci was highest ($1.91X10^{3}$ MPN 100 ml^{-1}) at the SIWWTP, which does not have secondary treatment but only uses primary treatment followed by UV disinfection. Secondary effluent from the HKWWTP had $3.47X10^{1}$ MPN 100 ml^{-1}, and R1 from the HOWWTP had $4.0X10^{0}$ MPN 100 ml^{-1} (Table 3).

Using the cultivation-based method (Enterolert®), concentrations of enterococci at the HKWWTP decreased from raw sewage by 44.85%, 99.91% and 99.99% after primary, secondary and UV disinfection treatments respectively. At the HOWWTP enterococci concentrations decreased by 72.15%, 95.76% and 100% after primary, secondary and R1 treatments respectively. At the SIWWTP enterococci concentrations decreased by 95.82% and 99.85% after primary and UV treatments respectively (Table 3).

4.3.2 Enterococci and HBAC concentrations in sewage using qPCR

Concentrations of enterococci in the influent samples measured using qPCR were not significantly different from those derived using the cultivation-based method ($p=0.702$) and remained within an order of magnitude throughout the treatment process, except for those observed at the post UV treatment at the SIWWTP, in which enterococci concentrations determined by qPCR exceeded concentrations of enterococci determined by the cultivation based test two orders of magnitude difference (Table 3), likely due to the UV effect on

cultivability. In regards to treatment efficiency as well as *Enterococcus* and HBAC concentrations on the outfalls, concentration of enterococci decreased by 99.95% and HBAC concentration decreased by 99.99% from primary influent to final effluent at the HKWWTP, Enterococci concentration decreased by 99.92% and HBAC concentrations decreased by 98.28% from primary influent to final effluent at the HOWWTP. Enterococci concentrations decreased by 91.24% from primary influent to primary effluent and 89.65% from primary effluent to UV at the SIWWTP. HBAC concentrations decreased by 79.13% and 81.42% respectively for the same treatment stages at the Sand Island plant (Table 3).

Average HBAC concentrations in primary influent were highest at the SIWWTP ($3.62X10^{9}$gc 100 ml^{-1}), followed by HKWWTP ($3.28X10^{9}$gc 100 ml^{-1}) and HOWWTP ($8.97X10^{8}$gc 100 ml^{-1}). This pattern was the same for enterococci. Concentrations of enterococci in primary influent were lower than those of HBAC at each treatment stage (primary, secondary and tertiary treatment) at all plants (Table 3). At the HKWWTP average concentrations measured using both methods had the same order of magnitude, at the HOWWTP the average concentration as measured by qPCR was lower by one order of magnitude than that measured using the cultivation method, and at the SIWWTP average concentrations using both methods were within the same order of magnitude (Table 3).

HBAC concentrations in the influent samples were significantly higher than when compared to concentrations of enterococci by cultivation ($p=0.004$) and qPCR ($p<0.01$). The concentration ratio of HBAC to enterococci in primary influent was 14,713:1, 11,991:1 and 860:1 at HKWWTP, HOWWTP and SIWWTP respectively. This indicates that the HBAC marker is well suited to determine whether enterococci are from sewage, as its concentrations exceed those of enterococci in wastewater. It should always be present when sewage-borne enterococci are detected.

Table 3.WWTP's concentrations and % reduction of enterococci by cultivation, molecular methods and HBAC concentrations.

Sample type	Sample Size	Enterococci (cultivation) MPN 100 ml^{-1}		Enterococci (qPCR) cells 100 ml^{-1}		HBAC gc 100 ml^{-1}	
		Average (min-max)	Reduction (%)	Average (min-max)	Reduction (%)	Average (min-max)	Reduction (%)
HKWWTP Influent	3	$4.18X10^5$ ($2.92X10^5$-$4.88X10^5$)	0	$2.23X10^5$ ($1.86X10^5$-$2.80X10^5$)	0	$3.28X10^9$ ($2.01X10^9$-$4.69X10^9$)	0
Primary treated	3	$2.30X10^5$ ($1.09X10^5$-$3.44X10^5$)	44.85	$5.64X10^4$ ($3.64X10^4$-$9.19X10^4$)	74.69	$1.91X10^9$ ($1.41X10^9$-$2.61X10^9$)	41.71
Secondary treated	3	$3.80X10^2$ (<$1.00X10^2$-$5.20X10^2$)	99.91	$2.58X10^2$ ($1.08X10^2$-$5.32X10^2$)	99.88	$5.14X10^5$ ($2.68X10^5$-$7.78X10^5$)	99.98
UV	3	$3.47X10^1$ (<1-$6.20X10^1$)	99.99	$1.20X10^2$ ($8.48X10^1$-$1.42X10^2$)	99.95	$2.72X10^5$ ($1.56X10^5$-$3.44X10^5$)	99.99
HOWWTP Influent	3	$3.46X10^5$ ($2.75X10^5$-$4.26X10^5$)	0	$7.48X10^4$ ($4.69X10^4$-$9.76X10^4$)	0	$8.97X10^8$ ($8.11X10^8$-$1.10X10^9$)	0
Primary treated	3	$9.64X10^4$ ($6.89X10^4$-$1.27X10^5$)	72.15	$6.54X10^4$ ($4.02X10^4$-$1.04X10^5$)	12.54	$6.10X10^8$ ($5.25X10^8$-$8.31X10^8$)	31.96
Secondary treated	3	$1.47X10^4$ ($5.44X10^3$-$2.38X10^4$)	95.76	$4.10X10^3$ ($3.59X10^3$-$4.68X10^3$)	94.51	$1.32X10^7$ ($1.96X10^6$-$2.16X10^7$)	98.53
R1	3	4.00 (<1-$1.00X10^1$)	100.0	$6.19X10^1$ ($1.07X10^1$-$1.15X10^2$)	99.92	$1.54X10^7$ ($2.36X10^4$-$7.65X10^7$)	98.28
SIWWTP Influent	3	$1.25X10^6$ ($7.98X10^5$-$1.94X10^6$)	0	$4.21X10^6$ ($3.03X10^6$-$5.16X10^6$)	0	$3.62X10^9$ ($2.53X10^9$-$4.61X10^9$)	0
Primary treated	3	$1.78X10^5$ ($1.57X10^5$-$2.10X10^5$)	85.82	$3.69X10^5$ ($2.19X10^5$-$5.20X10^5$)	91.24	$7.57X10^8$ ($5.12X10^8$-$9.48X10^8$)	79.13
UV	3	$1.91X10^3$ ($9.70X10^2$-$2.59X10^3$)	99.85	$4.36X10^5$ ($7.79X10^4$-$6.73X10^5$)	89.65	$6.73X10^8$ ($2.97X10^8$-$9.73X10^8$)	81.42

4.4 Task 4: To evaluate practicality of the test, utilize the assay to evaluate water quality on a selected beach in parallel with molecular and cultivation-based *Enterococcus* assays

4.4.1 Enterococci and HBAC concentrations in beach samples

Enterococci was undetectable in all the Kahanamoku (n=15) and Ala Moana Bowls (n=15) samples using cultivation-based methods. Lagoon samples (n=15) had three positives, but all under the state standard of 130 MPN 100 ml^{-1}(ranging from 10-31) (

	Flow (mgd)	
WWTP	2012	2020
Sand Island	58.33	92.60
Honouliuli	25.22	32.80
Waianae	3.33	3.50
Wahiawa	1.64	1.89
Pa'ala'a Kai	0.08	0.14
Kailua	10.29	13.40
Waimanalo	0.54	0.62
Kahuku	0.18	0.15
Laie	0.44	0.63
Hawaii Kai	0.51	0.38

*Table A.**2***).

The molecular method detected enterococci more often than Enterolert®. Enterococci were detected eleven times at Kahanamoku beach, thirteen times at Lagoon and nine times at Kahanamoku beach. However, only one of the detections was above quantification limits (167 cells 100 ml^{-1} at Lagoon) and the result wasn't confirmed using the cultivation method (

Table A.3).

HBAC was detected one time at Ala Moana Bowls, four times at Lagoon and one time at Kahanamoku Beach. Only one of the detections was above quantification limits (Lagoon) (

Table A.*4*).

4.4.2 Time between sample collection and results posting for beach samples

For beach samples, it took more than three hours from sample delivery by SFOC to results posting three out of fifteen times. The average results posting three out of fifteen times. The average time for this process was three hours and two minutes. The minimum time was one two minutes. The minimum time was one hour and 52 minutes and this was accomplished on two separate occasions. The earliest time for two separate occasions. The earliest time for results posting was 8:52 am (

Table A.5).

4.4.3 Laboratory quality control for the beach samples

R^2 and efficiency of the standards used to quantify enterococci concentrations in beach samples ranged from 0.964 to 0.991 and 81.70 to 112.20 respectively. R^2 and efficiency for the BacHF183/R287TaqMan assay were 0.980-0.998 and 90.20-105.10 respectively (Table A.6).

4.5 Task 5: To determine correlation between the modified molecular method for *Enterococcus* and Enterolert® cultivation-based test, evaluate water quality in Ala Wai Canal

4.5.1 Enterococci and HBAC concentrations in Ala Wai samples

Concentrations of enterococci determined using molecular methods were significantly higher than concentrations of enterococci determined higher than concentrations of enterococci determined using cultivation based methods (p=0.004). In the Ala Wai samples (n=60), the In the Ala Wai samples (n=60), the geometric mean (GM) concentration of enterococci detected using the molecular method was 309 cells using the molecular method was 309 cells 100 ml^{-1} when all five sites were pooled (

Table A. 7)**. The highest GM of concentration was 339 cells 100 ml^{-1} and the lowest 249 cells 100 ml^{-1} (**

Table A. 7)**. GM of each individual site for enterococci concentrations were lower at all sites using the cultivation-based method than the molecular method. The highest GM for enterococci concentration measured using the cultivation method was 214 MPN 100 ml^{-1}, and**

the lowest was 158 MPN 100 ml^{-1}(

Table A. 7).

Average concentrations of HBAC were significantly lower than those found for enterococci using the molecular-based method (p=0.005), but not different from concentrations of enterococci estimated by cultivation based methods (p=1.000). This in contrast with the HBAC to enterococci ratio we determined for Hawaii sewage samples, where concentrations of HBAC exceed several orders of magnitude concentrations of enterococci. Collectively this suggests that substantial amount of the enterococci seen in the canal were not of sewage origin. The highest GM concentration of HBAC was 211 gc 100 ml^{-1} and the lowest 105 gc 100 ml^{-1} (*Table A. **8***).

Overall, 25 out of 60 (42%) Ala Wai samples were positive for HBAC (Table A. 8), 49 out of 60 (82%) were positive for enterococci using molecular methods (

Table A. 7) and all the samples were positive for enterococci using the cultivation method (

Table A. 7). However, only 29 out of 60 (48%) samples exceeded the 130 MPN 100 ml^{-1}(using the cultivation method) threshold recommended by EPA (

Table A. 7). 24 of the 60 samples were positive for both HBAC and enterococci using molecular methods (

Table A. 7, Table A. 8).

61% of the Ala Wai samples exceeded the enterococci beach closure threshold as measured by both Enterolert® and molecular methods. The other 39% of the samples would have exceeded enterococci's threshold only by molecular methods (Table 4). 33% of the samples indicating unsafe water for swimming by both enterococci methods contained HBAC. 63% of samples that exceeded the beach posting threshold for enterococci as measured by molecular methods were also positive for HBAC. Only samples that did not have substantial interference (as identified by $\Delta C_t \leq 3.3$), were used in this analysis.

Table 4: Percentage of samples that crossed beach closure threshold using the two analytical methods (%)

		Method 1611	
		Exceeded	Not exceeded
Enterolert®	Exceeded	49	0
	Not exceeded	39	12

4.5.2 Correlation of HBAC and enterococci

HBAC concentrations did not correlate with concentrations of enterococci determined by molecular ($R^2 = 0.13$) and cultivation-based methods ($R^2 = 0.16$) (*Figure 3*).

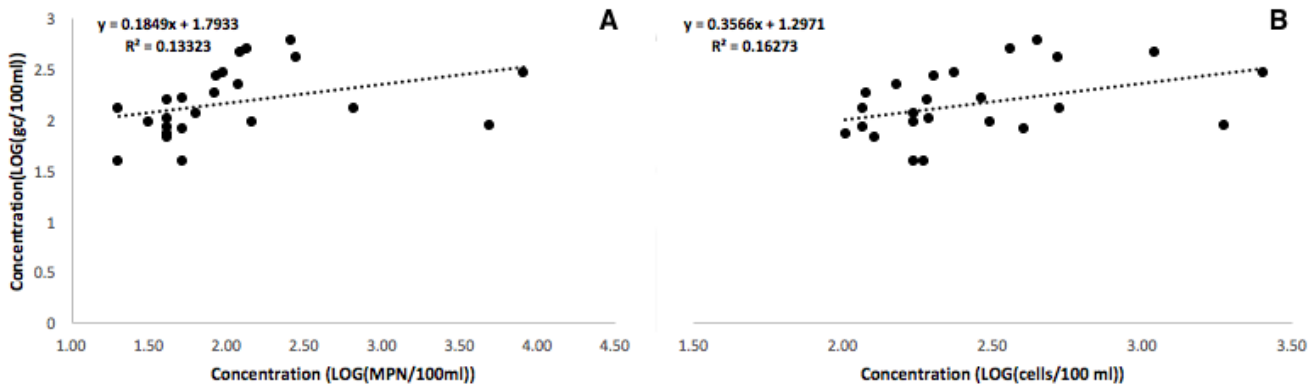

Figure 3: Enterococci by cultivation-based (A) and rapid molecular methods (B) versus HBAC.

4.5.3 Salinity and concentrations of HBAC and enterococci

Moderate correlation was found between enterococci and salinity using cultivation method (R^2 =0.45) and weak correlation using molecular methods (R^2 =0.21) (*Figure **4***).

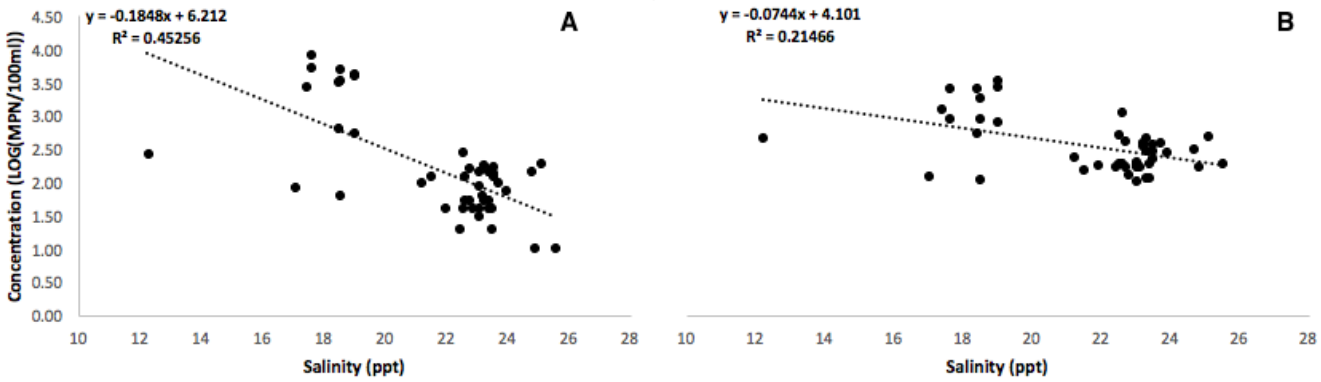

Figure 4:Salinity versus Enterococci by cultivation-based (A) and rapid molecular methods (B).

Very weak correlation was also found between HBAC and salinity (R^2 =0.11) (*Figure 5*).

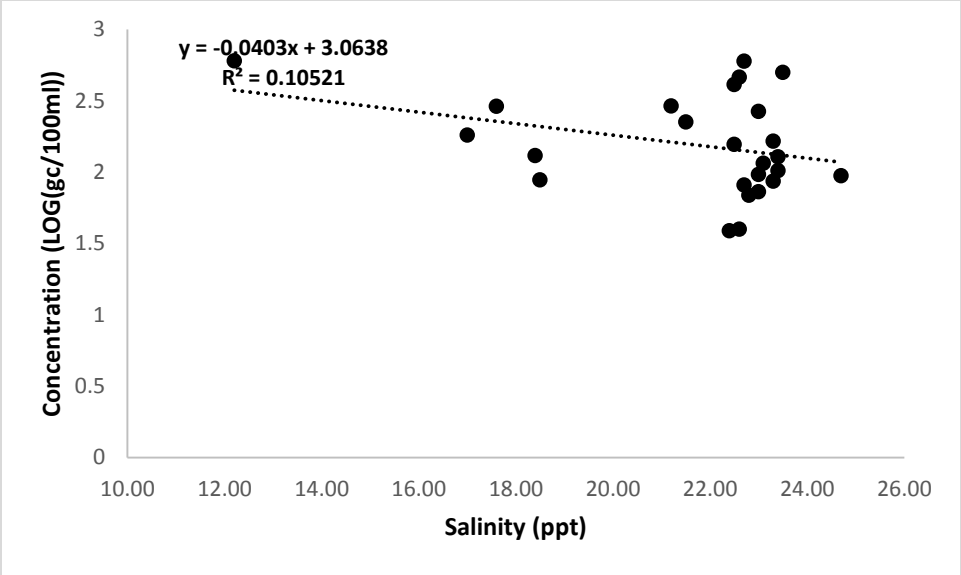

Figure 5: Salinity versus HBAC.

4.5.4 Correlation and index of agreement cultivation-based and molecular methods

Strong correlation (R^2=0.76) and index of agreement (IA=0.78) were found between enterococci concentration estimates derived by molecular and cultivation-based methods. The coefficient of correlation (R^2) and IA were both above values required by EPA for validation of alternative water quality methods (0.6 and 0.7 respectively) (Figure 6) (USEPA, 2014).

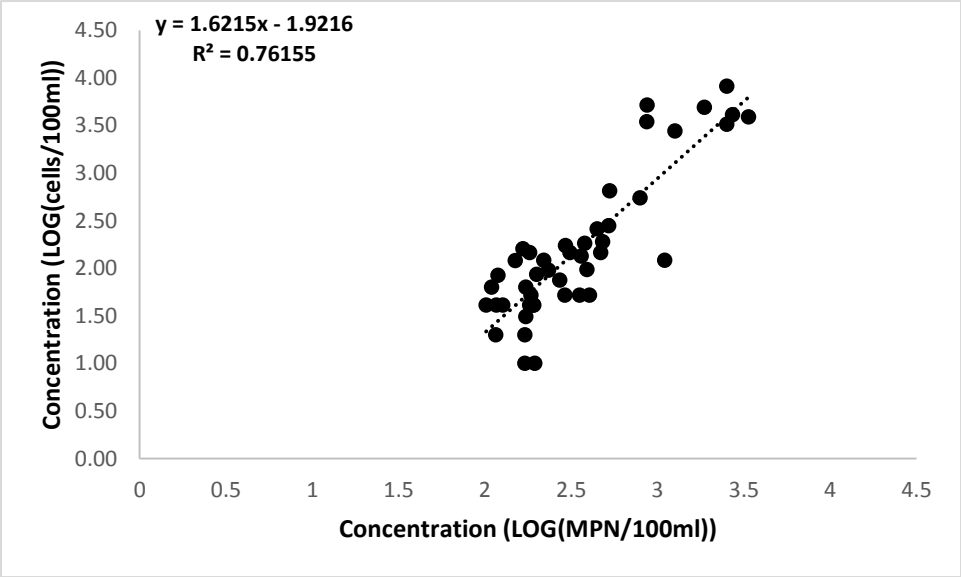

Figure 6: Log concentrations of enterococci using cultivations-based (x-axis) and rapid molecular methods (y-axis)

CHAPTER 5

DISCUSSION

Rapid methods outlined here could be used for timely water quality evaluations as the new method we tested takes less time than the cultivation methods currently used by the DOH.

5.1 Task 1: Setup of the qPCR assays for enterococci and HBAC to determine if our laboratory procedures are working properly (

Table *1*).

LLD for enterococci and HBAC was twenty cells 100 ml^{-1} and twenty gene copies (gc) 100 ml^{-1} respectively. The LLQ for both assays were five cells or gc per 25 µl PCR reaction, which represents 100 cells or gc per sample (100 ml). LLD and LLQ for enterococci were lower 1.00 $X10^2$ cells 100 ml^{-1} than Hawai'i's standards (130 cells 100 ml^{-1}), which implies that enterococci can be detected and quantified using molecular tests as it was set up in our experiments. Efficiency for both enterococci and HBAC standards were between 90% to 110% and R^2 were above 0.990 which indicates good assay performance (USEPA, 2012; USEPA, 2013) (Figure A. 6).

5.2 Task 2: Validate the HBAC assay for specificity and sensitivity.

Enterococci were detected in all species and in 52 out of 64 animal samples tested, suggesting that this indicator is not human specific in Hawai'i. The average concentrations of enterococci in the samples was of the same order of magnitude (10^6 cells 100 ml^{-1}) for all animal feces except mongoose and rat, which showed average concentrations one order of magnitude greater (10^7 cells 100 ml^{-1}).

In this study HBAC was only found in chicken, dog, human and mongoose samples. Human and dog feces samples showed the highest sensitivity (100% and 91% respectively), however HBAC was more concentrated in human samples and average concentrations were two orders of magnitudes higher than dog samples (averaging 10^{10} gc 100 ml^{-1}vs. 10^8 gc 100 ml^{-1}respectively). Previous studies also found that dog and human samples have high sensitivity (82% and 80% respectively) for BacHF183/R287TaqMan (Kirs et al., 2016; Boehm et al., 2013). Chicken and mongoose samples did contain HBAC marker (40% and 20% respectively), but at substantially lower concentrations (averaging 10^4 gc 100 ml^{-1} and 10^4 gc 100 ml^{-1}) suggesting that when this marker is found in environmental waters its source was not these two animals. Previous studies done in the mainland United States found HBAC in deer, farmed and wild pig and dog feces (Boehm et al., 2013). In a more recent study done in Hawai'i, HBAC was present in dog, mongoose and cat samples (Kirs et al., 2016). Cross-reactivity was more significant in dog samples than in any of the other animals tested, suggesting that they might be the biggest non-human source of environmental HBAC. Collectively this suggests that pigs, rats, cats, chickens and mongoose feces are not the most important cross-reactivity concerns due to the low concentrations and inconsistent recovery of HBAC in their feces. Cross-reactivity must be considered in MST practice because the HBAC marker is found in animals and can falsely implicate sewage as the contamination source. The overall specificity of the marker (BacHF183/R287TaqMan) was comparable to that found for the older version of the marker

(BacHF183/BFDrevTaqMan) studied by Kirs et al. (2016) with 74%.

5.3 Task 3: Compare concentrations of both assays in sewage

This study suggests that sewage-borne HBAC would be more easily detected in the environment than enterococci as the concentrations of HBAC were significantly higher than when compared to concentrations of enterococci determined by cultivation ($p=0.004$) and qPCR ($p<0.001$) methods (Table 3). Moreover, HBAC is an obligate anaerobe implying that it can only come from sewage and not from extra-host environments. (Haugland, 2010). HBAC concentrations in the WWTPs primary influent was at least two logs higher than that seen in dog feces (the highest non-human concentrations measured in this study), which implies that detection in the environment most likely would be attributable to human sewage and less likely to animals. This study found HBAC in all sewage samples (100% sensitivity), which replicates results found in other states (Layton et al., 2013). Lastly, in Kirs et al. (2016) HBAC concentrations had a ten-fold higher or same order of magnitude as enterococci in primary influent. In this study, HBAC concentrations in primary influent were at least three logs higher than concentrations of enterococci (Table 3). This suggests that the newer version of the assay used in this study might be better suited for use in Hawaii, although variability of marker concentrations over time might also explain the difference between the earlier and this study.

5.4 Task 4: To evaluate practicality of the test, utilize the assay to evaluate water quality on a selected beach in parallel with molecular and cultivation-based *Enterococcus* assays

80% of the beach samples were analyzed and results posted by the SFOC within three hours. The minimum time was one hour and 52 minutes and that was achieved on two separate occasions. The earliest time that results were posted was at 8:52 am (

Table *A.5*). This means that if samples were delivered by 6:00 am posting could occur at 7:52 am before the public arrives at the beach, characterizing the method as rapid. In general, time to post beach sample results decreased over the course of the experiment, except on day nine which was an outlier when real time approval of the results was not possible. Our assay took one hour and four minutes to complete, compared to the EPA recommended protocol (> one hour and 40 minutes) and cultivation-based methods (≥24 hours).

In beach samples, enterococci were detected more often using molecular methods than the cultivation method. Using the cultivation-based method enterococci was detected in only three out of 45 beach samples collected and all of those were below the state standard. Using molecular methods, enterococci was detected in 33 out of 45 samples but only one was above the state standard (day seven at Lagoon). This could be because molecular methods detect viable but non-culturable (VBNC) and culturable cells, while the cultivation method detects strictly culturable cells. Because only one sample had values above EPA recommendation, beach closure decisions would not have been affected.

Nevertheless, detection of HBAC in five out of 45 beach samples may be indicative of sewage contamination.

5.5 Task 5: To evaluate correlation between rapid methods and Enterolert® tests, utilize the assay to evaluate water quality on Ala Wai Canal in parallel with molecular and cultivation-based *Enterococcus* assays

As already mentioned, in order to be approved by the EPA, the alternative method that we tested must agree with the currently approved EPA method. More specifically the Index of Agreement (IA) and the correlation coefficient (R^2) between the two methods must be equal or exceed 0.7 and 0.6 respectively (USEPA, 2014). While we were unable to compare, beach samples using both methods due to low concentrations of enterococci, for the Ala Wai Canal samples both IA (0.78) and R^2 (0.76) exceeded the EPA benchmarks, implying that the modified, more rapid version of the enterococcus molecular method can be used in Hawai'i. Average concentrations of enterococci in the Ala Wai Canal were higher for all sites using molecular methods compared to the cultivation-based method. Both the cultivation-based and the molecular methods agreed on exceedance/non-exceedance of the notification threshold for 61% of the total samples collected. The other 39% indicated that beaches exceeded threshold value based on the molecular method, but did not exceed that value using the cultivation-based method. This suggests that the cultivation-based method used may be less conservative than the molecular method in detecting enterococci. It should be noted that molecular methods detect both viable and non-viable cells, whereas the cultivation-based method detects exclusively cultivable cells. Enterococci can enter a viable but not culturable (VBNC) state when encountering stressful conditions, this would make them undetectable by culture based methods which brings into question the adequacy of culture based methods regarding water quality and health risks (Gin and Goh, 2013). The health risk associated with VBNC has not be determined. However, recent epidemiological studies have demonstrated that molecular methods that measure DNA from enterococci (including those of VBNC) might be better predictors of health risk when compared to cultivation based methods (Wade et al., 2010). Our analyses indicated that the results from our cultivation-based methods and molecular methods for enterococci produced significantly different results ($p=0.004$). Differences among cultivation-based and molecular methods are usually observed when cultivation based counts are low, but this does not appear to be the case in this study as enterococci concentrations were elevated in the canal (Whitman et al., 2010). Lastly, people could be less exposed to water contamination if molecular methods are used instead of cultivation based methods.

HBAC was less frequently detected in the Ala Wai samples when compared to enterococci. Only 63% of samples that exceeded the beach posting threshold for enterococci as measured by molecular methods were also positive for HBAC. Furthermore, concentrations of enterococci in the Ala Wai Canal were similar or higher (depending from the method used) when compared to concentrations of HBAC. This is in contrast with sewage samples analyzed were concentrations of HBAC were significantly higher when compared to concentrations of enterococci. As stated earlier, collectively this suggests that the source of enterococci and HBAC differed (sewage, soils, etc), and therefore, as suggested by other studies (Hardina & Fujioka, 1994; Byappanhalli & Fujioka, 2003; Kirs et al., 2016; Cui et al., 2013), these two indicators are not interchangeable.

5.6 Should the new method be implemented in Hawai'i? Who benefits?

Previous studies have shown that people's recreational behavior is affected by environmental quality. Ironically, people can change the environment by their recreational behavior (Pendleton et al., 2000). People's varying perceptions of environmental quality can be

explained by socio-economic status, cultural ties, and previous experiences (Bird, 1996). There are major societal downsides to incorrect beach posting and inaccurate water quality information. California has one of the most established recreational water quality monitoring programs in the United States. Several epidemiological studies have been conducted at Californian beaches (Colford et al., 2007). Despite having a highly protective recreational water quality program, many Californians have a negative perception of ocean water quality (Pendleton et al., 2000). On the other hand, some bathers disregard beach posting as signs are routinely posted after rain events rather than when there is a measurable risk (Griffith, personal communications). These attitudes are also seen in Hawai'i as people often do not have confidence in the methods utilized to determine beach water quality. Application of molecular sewage markers, as outlined in this paper, could decrease unnecessary beach postings in Hawai'i, as beaches would not be posted solely based on concentrations of enterococci from any and all sources, but only based on the detection of actual sewage-borne contaminants.

Hawai'i has numerous beaches which are open year around. The beaches are a major attraction for visitors to the state. Frequent beach postings could result in negative economic impacts. At Lake Michigan beaches, each beach closure between 1998 and 2001 represented an estimated economic loss ranging from $1,274-37,030 per day (Rabinovici et al., 2004). While Hawai'i does not close beaches due to high levels of FIB, economic losses due to changes in people's perception and visits are possible. In addition to preventing unwarranted posting of beaches, application of the rapid methods outlined in this paper, could also allow the HIDOH to remove caution and warning signs earlier when there is bona-fide sewage contamination.

It is envisioned that rapid molecular tests, as outlined here, could eventually be adopted for beach notification purposes in Hawai'i. These rapid tests are already being evaluated and utilized at several beaches in the mainland United States and overseas (Wade et al., 2010). Rapid test protocols and related management decisions would ultimately lead to cleaner and healthier environments and result in enhanced ecosystem services (Smith et al., 1999). Better protection of public health is expected to lead to lower sewage-borne illness' rates and related costs to society.

CHAPTER 6

CONCLUSIONS

- HBAC can be used as a sewage tracer in Hawai'i, although cross-reactivity with a few animals needs to be considered (74% specificity and 100% sensitivity).
- The modified faster version of the rapid test for enterococci (Method 1611) we tested meets EPA guidelines for alternative water quality methods (R^2 =0.76 and IA=0.78).
- Dual assay (enterococci and HBAC) can be applied in a timely fashion (less than two hours to report).
- 61% of the samples tested for enterococci in Ala Wai Canal would have resulted in the same beach posting decisions using cultivation based and molecular methods. If the Ala Wai Canal were a beach 39% of the samples would have had that beach remain open using cultivation-based methods but closed using rapid methods.
- Sewage is not likely the main source of enterococci in the Ala Wai Canal.
- From the Ala Wai Canal samples that exceeded for enterococci based on molecular methods, 63% of them were positive for HBAC, suggesting that in several samples enterococci detected was not of sewage origin.
- When measuring enterococci, cultivation-based methods might be less conservative than molecular methods. The health risk implications of this are unclear.

APPENDIX A

Table A. 1: WWTPs of O'ahu and respective flows.

	Flow (mgd)	
WWTP	2012	2020
Sand Island	58.33	92.60
Honouliuli	25.22	32.80
Waianae	3.33	3.50
Wahiawa	1.64	1.89
Pa'ala'a Kai	0.08	0.14
Kailua	10.29	13.40
Waimanalo	0.54	0.62
Kahuku	0.18	0.15
Laie	0.44	0.63
Hawaii Kai	0.51	0.38

Table A.2: Enterococci values for beach sites using cultivation-based method.

	IDEXX (MPN 100 ml^{-1})		
Day	Bowls	Lagoon	Kahanamoku
Day 1	0	10	0
Day 2	0	0	0
Day 3	0	0	0
Day 4	0	0	0
Day 5	0	0	0
Day 6	0	31	0
Day 7	0	0	0
Day 8	0	0	0
Day 9	0	0	0
Day 10	0	0	0
Day 11	0	20	0
Day 12	0	0	0
Day 13	0	0	0
Day 14	0	0	0
Day 15	0	0	0

Table A.3:Enterococci data reported to Surfrider.

	Enterococci (cells 100 ml^{-1})		
Day	Bowls	Lagoon	Kahanamoku
Day 1	Detected <100	Detected <100	Not Detected <100
Day 2	Detected <100	Detected <100	Detected <100
Day 3	Detected <100	Detected <100	Not Detected <100
Day 4	Detected <100	Detected <100	Detected <100
Day 5	Detected <100	Detected <100	Detected <100
Day 6	Detected <100	Detected <100	Detected <100
Day 7	Detected <100	Detected 167	Detected <100
Day 8	Detected <100	Detected <100	Detected <100
Day 9	Not Detected <100	Not Detected <100	Not Detected <100
Day 10	Not Detected <100	Not Detected <100	Detected <100
Day 11	Not Detected <100	Detected <100	Not Detected <100
Day 12	Detected <100	Detected <100	Detected <100
Day 13	Detected <100	Detected <100	Not Detected <100
Day 14	Detected <100	Detected <100	Detected <100
Day 15	Not Detected <100	Detected <100	Not Detected <100

Table A.4: HBAC data reported to Surfrider.

	HBAC (gc 100 ml^{-1})		
Day	Bowls	Lagoon	Kahanamoku
Day 1	non-detected, <1000	non-detected, <1000	non-detected, <1000
Day 2	detected, <1000	detected, <1000	detected, <1000
Day 3	non-detected, <1000	non-detected, <1000	non-detected, <1000
Day 4	non-detected, <1000	non-detected, <1000	non-detected, <1000
Day 5	non-detected, <1000	detected, >1000	non-detected, <1000
Day 6	non-detected, <1000	detected, <1000	non-detected, <1000
Day 7	non-detected, <1000	non-detected, <1000	non-detected, <1000
Day 8	non-detected, <1000	non-detected, <1000	non-detected, <1000
Day 9	non-detected, <1000	non-detected, <1000	non-detected, <1000
Day 10	non-detected, <1000	non-detected, <1000	non-detected, <1000
Day 11	non-detected, <1000	non-detected, <1000	non-detected, <1000
Day 12	non-detected, <1000	non-detected, <1000	non-detected, <1000
Day 13	non-detected, <1000	non-detected, <1000	non-detected, <1000
Day 14	non-detected, <1000	detected, <1000	non-detected, <1000
Day 15	non-detected, <1000	non-detected, <1000	non-detected, <1000

Table A.5: Time taken from sample delivery to results posting.

Day	Arrived	Finished	Total
Day 1	8:26	11:18	2:52
Day 2	8:05	12:31	4:26
Day 3	8.00	9:52	1:52
Day 4	7:40	10:51	3:11
Day 5	8:00	10:38	2:38
Day 6	8:15	11:05	2:50
Day 7	6:30	8:52	2:22
Day 8	7:45	9:48	2:03
Day 9	8:15	17:10	8:55
Day 10	7:51	10:36	2:45
Day 11	7:51	9:58	2:07
Day 12	8:15	10:40	2:25
Day 13	7:25	10:17	2:52
Day 14	7:30	9:50	2:20
Day 15	7:55	9:47	1:52

Table A.6: R^2 and efficiency from qPCR reactions on beach and Ala Wai samples.

		R^2	Efficiency
		Average (min-max)	Average (min-max)
Ala Wai	Enterococci	0.985 (0.952-0.996)	86.57 (79.1-106.7)
	HBAC	0.985 (0.953-0.998)	92.08 (80.3-102.8)
Beach	Enterococci	0.984 (0.964-0.991)	97.45 (81.70-112.20)
	HBAC	0.991 (0.980-0.998)	95.83 (90.20-105.10)

Table A. 7: Enterococci concentrations in Ala Wai Canal using qPCR (cells 100 ml^{-1}) and Enterolert (MPN 100 ml^{-1}) methods.

	Site 1		Site 2		Site 3		Site 4		Site 5	
Day	qPCR	Enterolert	qPCR	Enterolert	qPCR	Enterolert	qPCR	Enterolert	qPCR	Enterolert
Day 1	FR	146	FR	110	FR	132	FR	197	FR	75
Day 2	2721	4,106	3,374	3,872	869	3,448	2,518	3,255	1,874	4,882
Day 3	107	576	530	651	792	548	73	544	FR	604
Day 4	FR	19,863	870	5,172	1,263	2,755	2,519	8,164	FR	3,255
Day 5	182	146	167	160	379	183	235	95	482	189
Day 6	311	146	200	86	523	279	1,101	121	48	98
Day 7	362	134	150	120	119	84	447	259	FR	657
Day 8	190	41	294	173	182	41	110	63	FR	30
Day 9	469	146	391	97	219	121	100	52	272	75
Day 10	FR	74	354	52	192	41	291	52	173	31
Day 11	171	20	117	41	195	10	116	20	173	63
Day 12	404	52	185	52	127	41	102	41	170	10
Geometric Mean	321.5	213.6	339.3	201.2	322.2	158.2	303.7	180.4	249.4	164.2

*FR indicates wells that did not have DNA amplification (reaction failed)

Table A. 8: HBAC concentrations in Ala Wai Canal.

	HBAC (gc 100 ml^{-1})				
copies/rxn	Site 1	Site 2	Site 3	Site 4	Site 5
Day 1	FR	598.41	FR	FR	FR
Day2	ND	ND	ND	ND	87.98
Day3	ND	130.06	ND	ND	FR
Day 4	FR	ND	ND	288.83	FR
Day 5	ND	ND	ND	289.93	ND
Day 6	93.91	266.13	410.08	460.27	ND
Day 7	499.31	223.61	181.63	599.71	FR
Day 8	156.34	ND	ND	ND	FR
Day 9	ND	ND	ND	ND	ND
Day 10	FR	ND	102.45	164.04	96.15
Day 11	38.72	86.07	ND	127.86	115.29
Day 12	81.01	39.68	68.84	72.53	ND
Geometric Mean	118.12	119.40	152.39	211.17	105.28

Table A.9: Salinity at Ala Moana Bowls, Lagoon, Kahanamoku Beach and Ala Wai sites.

Salinity (ppt)								
	Beach Sites			Ala Wai Sites				
Day	Bowls	Lagoon	Kahanamoku	1	2	3	4	5
Day 1	29.9	30.1	30.3	22.3	22.7	22	20	22.3
Day 2	30.2	30.1	30.5	19	19	18.5	18.4	18.5
Day 3	30.2	30.4	30.3	19.2	18.4	19	20.5	20.3
Day 4	30.4	29.9	30.6	11.7	17.6	17.4	17.6	19.5
Day 5	30	30.3	30.5	23	22.7	23.2	21.2	25.1
Day 6	30.3	30.5	30.5	24.7	23	22.5	22.6	24.6
Day 7	29.8	30.3	30.4	23.5	21.5	17	12.2	10.5
Day 8	30.3	30.5	30.6	22.5	23.5	21.9	18.5	25.5
Day 9	30.5	30.7	30.8	23.3	23.7	23.5	23.6	23.9
Day 10	29.7	30	30.1	23.3	23.2	23.4	23.3	23
Day 11	30	30	29.9	22.4	23.3	25.5	23.4	23.1
Day 12	29.8	30	30.1	22.7	22.6	22.8	23	24.8
Day 13	29.9	30	30.1	-	-	-	-	-
Day 14	30.5	30.3	30.5	-	-	-	-	-
Day 15	30.6	30.7	30.9	-	-	-	-	-

Table A.10: Dates of beach and Ala Wai Canal sample collection.

	Beach	Ala Wai
Day 1	6/26/17	7/17/17
Day 2	6/27/17	7/18/17
Day 3	6/28/17	7/19/17
Day 4	6/29/17	7/20/17
Day 5	6/30/17	7/21/17
Day 6	7/3/17	7/22/17
Day 7	7/4/17	7/24/17
Day 8	7/5/17	7/25/17
Day 9	7/6/17	7/26/17
Day 10	7/7/17	7/27/17
Day 11	7/10/17	7/28/17
Day 12	7/11/17	7/29/17
Day 13	7/12/17	-
Day 14	7/13/17	-
Day 15	7/14/17	-

APPENDIX B

Figure A.1: QPCR machine

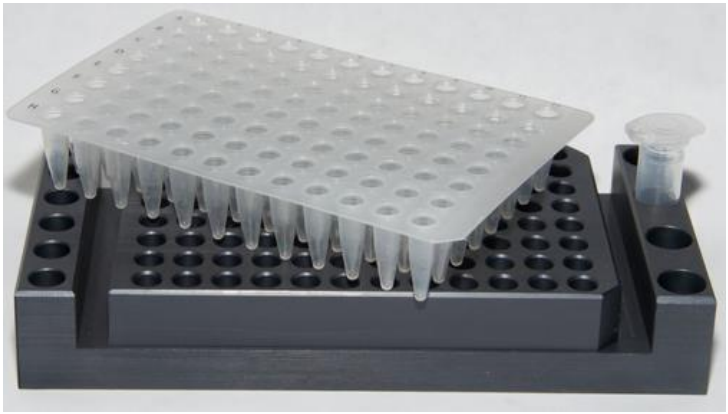

Figure A.2: 96-well plate

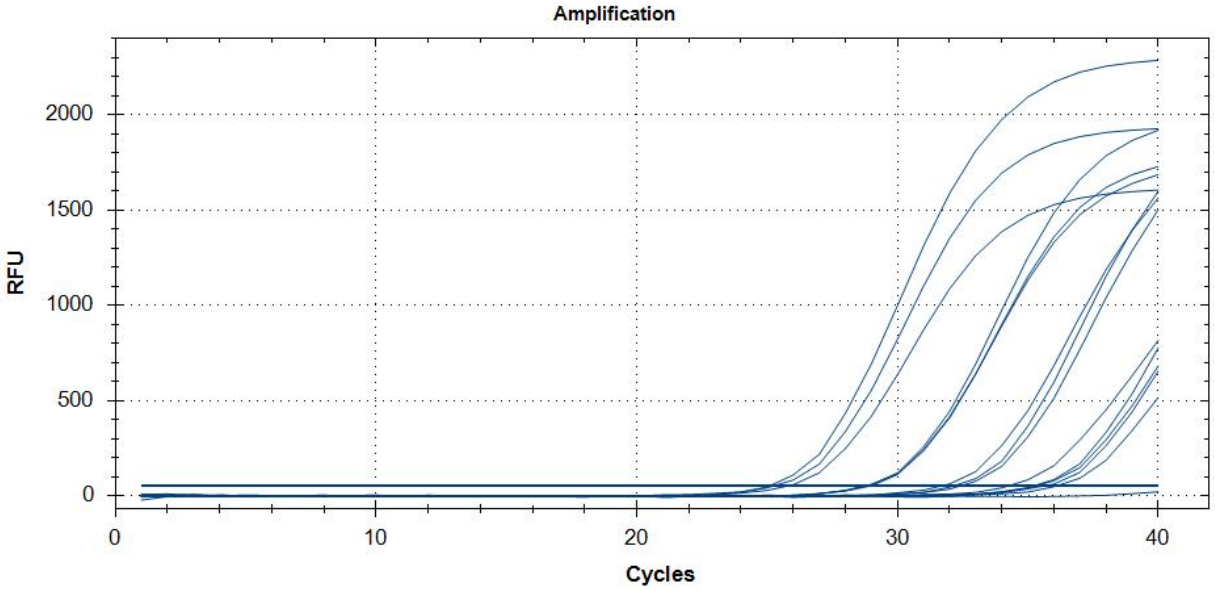

Figure A. 3: Example of a CFX96™Real-Time System PCR amplification curve. On the x-axis is the number of amplification cycles and on the y-axis the fluorescence intensity.

Figure A. 4: Beach sites selected: Ala Moana Bowls (1), Beach Lagoon (2) and Kahanamoku Beach (3).

Figure A. 5: Ala Wai Canal sites (1-5).

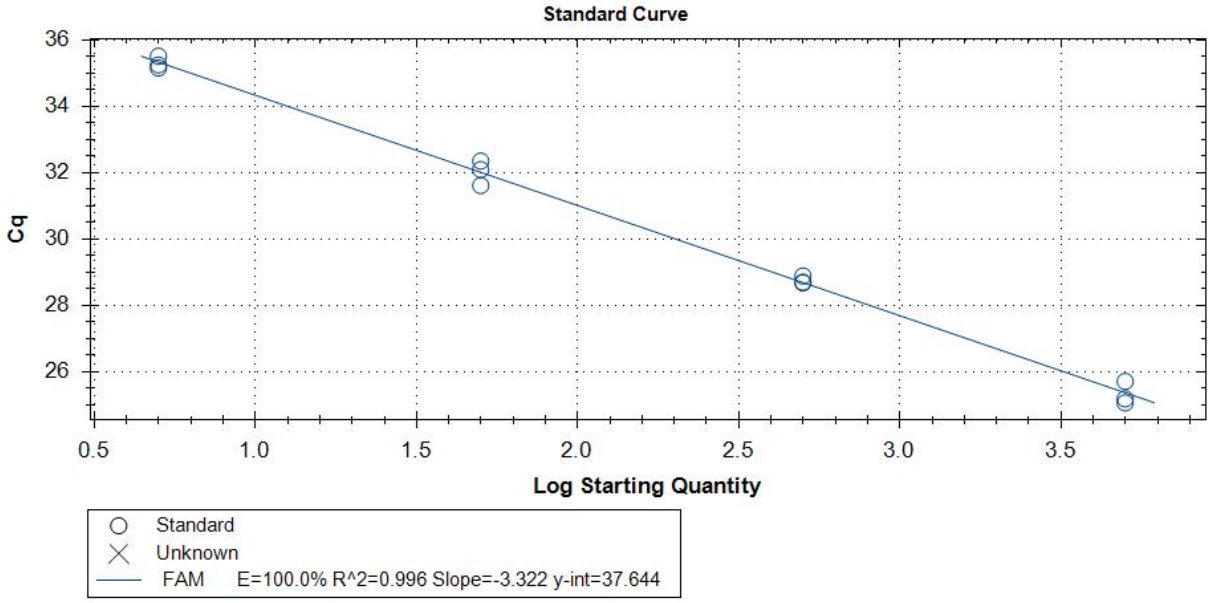

Figure A. 6: Standard curve for enterococci.

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