ENTEROCOCCI IN HAWAIʻI BEACH WATER

A THESIS SUBMITTED FOR PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR THE DEGREE OF

BACHELOR OF SCIENCE

IN

GLOBAL ENVIRONMENTAL SCIENCE

MAY 2025

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

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For the health of the ʻāina and its people, and to clean water for all.

ACKNOWLEDGEMENTS

Thank you to the Hawai‘i Department of Health for providing funding and water samples and to the ASGPB Sequencing Lab for sequencing our DNA.

Thank you to my mentor, Dr. Marek Kirs, for all of his time, patience and care as I delved into the world of microbiology. Dr. Kirs dedicated countless hours to my project, teaching me everything I know in the lab, offering me invaluable opportunities and always prioritizing my mental well-being. He has not only fostered my growth as a scientist, but most importantly showed me what it means to be a good person.

And most of all, thank you to my parents, whose love and support gave me the strength to grow into the woman I am today. Your unwavering belief in me has carried me further than words will ever be able to express.

I am so immensely grateful to everyone who has been a part of my journey so far. I am filled with excitement for what life may bring next, and with hope for the future of our planet.

ABSTRACT

Enterococci concentrations are used to develop water quality standards to protect recreational water users from sewage-borne pathogens. In Hawai‘i, the Enterolert® test (IDEXX Laboratories, Inc.) is commonly used to determine enterococci concentrations in coastal waters, with a threshold of 130 MPN per 100 mL triggering public health advisories by the Hawai‘i Department of Health (HDOH). This study evaluated the specificity of the Enterolert® test in Hawai‘i beach water samples and determined the presence of sewage contamination. A total of 49 enterococci-positive beach water samples were collected from 24 different beaches. Of these 49 samples, there were 85 enterococci-positive wells from Quanti-Trays®, of which DNA analysis revealed a false-positive rate of 11.76%, with non-*Enterococcus* species including *Clostridium tertium* (2.35%), *Vagococcus luciliae* (3.53%), *Streptococcus gordonii* (1.18%), *Bacillus* (1.18%), *Vagococcus bubulae* (1.18%), *Paenibacillus dendritiformis* (1.18%), and *Vagococcus fluvialis* (1.18%). Microbial source tracking using CrAssphage and the HF183 markers indicated traces of human sewage in 63.3% of enterococci-positive samples (n=49), with CrAssphage detected in 42.9% and HF183 in 36.7%. The source of enterococci in the remaining samples could not be determined. This study suggests that the Enterolert® Test exhibits higher false-positive rates in Hawai‘i than reported in temperate regions, with a substantial portion of enterococci detections resulting from non-human fecal sources. As a result, this may overestimate health risks in coastal waters and highlights the need for an alternative fecal indicator bacteria better suited to tropical environments.

Keywords: Enterococci, beach water, Enterolert® Test, water quality monitoring

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LIST OF ABBREVIATIONS AND SYMBOLS

BAV: Beach Action Value

BEACH Act: Beaches Environmental Assessment and Coastal Health Act

CFU: Colony-Forming Units

Cq: Cycle Threshold

CWB: Clean Water Branch

CWA: Clean Water Act

DNA: Deoxyribonucleic Acid

EPA: Environmental Protection Agency

FIB: Fecal Indicator Bacteria

gC: Gene Copies

HCl: Hydrochloric acid

HDOH: Hawai‘i Department of Health

MST: Microbial Source Tracking

MPN: Most Probable Number

PCR: Polymerase Chain Reaction

qPCR: Quantitative Polymerase Chain Reaction

RFU: Relative Fluorescence Units

STV: Statistical Threshold Value

UV: Ultraviolet

WWTP: Wastewater treatment plants

WRRC: Water Resources Research Center

°C: Degrees Celsius

mL: Milliliter

µL: Microliter

µM: Micromole

µm: Micrometer (Micron)

1.0 INTRODUCTION

1.1 ENTEROCOCCI AS A FECAL INDICATOR BACTERIA

Enterococci are gram-positive cocci classified among the lactic acid bacteria, within the clostridial branch (Naser, 2005). They are commensal bacteria that naturally inhabit the gastrointestinal tract of warm-blooded animals, including humans (Byappanahalli et al., 2012). Due to their abundance in human feces, they have been designated as fecal indicator bacteria (FIB), serving as a proxy for predicting the presence of fecal contamination in water bodies (Boehm et al., 2014). Among enterococci, *Enterococcus faecalis* and *Enterococcus faecium* are the most common species found in human fecal material (Lebreton et al., 2014).

Although enterococci are not considered to be a direct threat to healthy populations, their presence can indicate the occurrence of other harmful pathogens, particularly viruses that are a primary cause of recreational water illnesses (US EPA, 2025; Benjamin-Chung et al., 2017). While enterococci are widely used as an indicator of fecal contamination, they can also persist and multiply within environmental settings including sediments, beach sand, and vegetation. These are commonly referred to as "naturalized" FIBs, many of which may be non-enteric, meaning they do not originate from animal or avian sources (Devane et al., 2020). The presence of these non-enteric FIBs can lead to an overestimation of swimming-related health risks.

In Hawai‘i and other tropical regions, naturalized FIBs, including enterococci, tend to survive in soil and decomposing vegetation, particularly in areas with high volumes of plant matter (State of Hawai‘i Department of Health, 2024). Independent of their original source, enterococci often enter marine coastal environments following

periods of precipitation, primarily through agricultural runoff, stormwater, and sewage discharges (Boehm, 2014). As a result, heavy rainfall events are often associated with elevated FIB levels in coastal waters and short-term enterococci exceedances above 130 MPN/100 mL (Most Probable Number per 100 milliliters of water), making recreational use unsafe—notably when waters appear brown (Clean Water Branch, n.d.; Adolf et al., 2023). Exposure to waters where FIBs are present can increase the risk of gastrointestinal illnesses, especially among vulnerable populations. Therefore, routine monitoring of beach water for enterococci is essential to help mitigate health risks for recreational water users.

1.2 BEACH WATER QUALITY MONITORING IN HAWAIʻI

The Clean Water Act (CWA) was amended by the Beaches Environmental Assessment and Coastal Health Act of 2000 (BEACH Act) mandating that areas with coastal recreational waters adopt water quality standards for pathogens as outlined in CWA section 304(a) by the US Environmental Protection Agency (EPA). To support this requirement, the EPA provides grants to support beach monitoring programs that assess coastal water quality (State of Hawaiʻi Department of Health, 2024).

In Hawaiʻi, the Hawaiʻi Department of Health (HDOH), Clean Water Branch (CWB) is a recipient of the EPA BEACH Act funding and adheres to EPA performance criteria for monitoring. The Beach Action Value (BAV) of 130 MPN/100 mL is based on the Statistical Threshold Value (STV) for enterococci. When this threshold is exceeded, a Beach Notification is posted on the CWB Water Quality Notification and Advisories website to inform the public of potential health risks. Follow-up water samples are then

collected the next day with warning signs posted on affected beaches if the exceedance is confirmed. The BAV is based on the estimated risk of 36 cases of illness per 1,000 swimmers. The primary aim of the Hawai‘i Beach Monitoring Program is to limit gastrointestinal illness among recreational beach users. Beach monitoring frequency and priority are determined by following the Beach Tiering Criteria, which classifies beaches based on year-round recreational use, proximity to streams that flow through developed areas, history of sewage spills, high beach usage, economic and community significance, prior data showing elevated fecal indicator bacteria (FIB), and ease of public access (State of Hawai‘i Department of Health, 2024). Beaches with five or greater of these factors are considered to be Tier 1. Given that Tier 1 beaches are sampled more frequently, a higher number of enterococci detections is expected; however, the

Tier 1	Tier 1 beaches are considered "core" beaches and are ranked as such because of their economic and social importance to the state. Tier 1 beaches are heavily used and most are stationed by lifeguards. Tier 1 beaches may be threatened by some type of pollution. These beaches are given the highest monitoring priority.
Tier 2	Tier 2 beaches also include beaches which may be economically or socially important to the state but are less heavily used than Tier 1 beaches. Tier 2 beaches are currently monitored on a less frequent basis compared to Tier 1s due to resource constraints; however, the frequency may be increased as resources become available.
Tier 3	Tier 3 beaches are even less heavily used, do not have a history of high FIB concentrations, are less threatened than Tier 1 or Tier 2 beaches, and currently receive no routine monitoring due to the lower threat and usage level. Tier 3 beaches also include those beaches that may pose a safety hazard to the sampler.

concentration (MPN/100 mL) is ultimately more indicative or water quality conditions.

Table 1. Beach Tiering Criteria; Source: (State of Hawai‘i Department of Health, 2024).

The Water Resources Research Center (WRRC), Environmental Microbiology Lab at the University of Hawai‘i at Mānoa collaborates with the HDOH in routine beach

water quality monitoring. This includes analyzing beach water samples for enterococci (MPN/100 mL) and *Clostridium perfringens* (CFU/100 mL). Enterococci levels are measured using the Enterolert® Test (IDEXX Laboratories), while *C. perfringens* is detected using the mCP agar method, which involves filtering water samples through 0.45 µm pore-size filters and culturing them (Bisson et al., 1979). Colonies initially appear yellow and turn pink upon exposure to ammonia hydroxide vapor. While *C. perfringens* serves as a confirmatory water quality indicator, there is no EPA regulatory exceedance threshold, and results are not required to be publicly posted online (State of Hawai‘i Department of Health, 2018). However, the CWB generally considers a threshold value of <50 CFU/100 mL of marine water (State of Hawaii, 2012) as indicative of risk from sewage contamination. Unlike enterococci, *C. perfringens* is unable to reproduce in aerobic environments. However, it is a spore former, and its spores can survive for extended periods, and as a result, its presence does not necessarily indicate recent fecal contamination (Shibata et al., 2004). Laboratory analysis requires an incubation period of 24 hours for enterococci and 22-24 for *C. perfringens*, before results are reported to the HDOH. Consistent monitoring of both bacteria is essential for accurately assessing water quality and reducing water-borne illnesses.

1.3 THE ENTEROLERT® TEST BY IDEXX LABORATORIES

Various methods are used to enumerate enterococci levels in water, including culture-based techniques such as membrane filtration and molecular approaches involving deoxyribonucleic acid (DNA) extraction, amplification, and quantification. The Enterolert® Test (IDEXX Laboratories) is a U.S. EPA-approved, defined substrate

method that estimates enterococci levels using the Most Probable Number (MPN) per sample volume, delivering results within 24 hours of processing (US EPA, 2012). The test utilizes 4-methylumbelliferyl-β-D-glucoside, a nutrient-indicator substrate. When metabolized by enterococci through the enzyme beta-glucosidase, 4-methylumbelliferone and glucose are released, resulting in fluorescence under ultraviolet (UV) light (see Figures 1 and 2). This process selectively supports the growth of enterococci while antimicrobial agents inhibit non-target bacteria from metabolizing 4- methylumbelliferyl-β-D-glucoside (Chen, 1995).

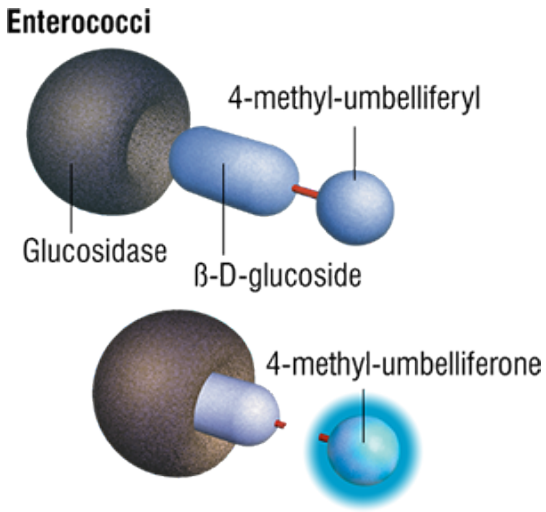

Figure 1. Visual representation of the Enterolert® Test mechanism that results in fluorescence under UV light.

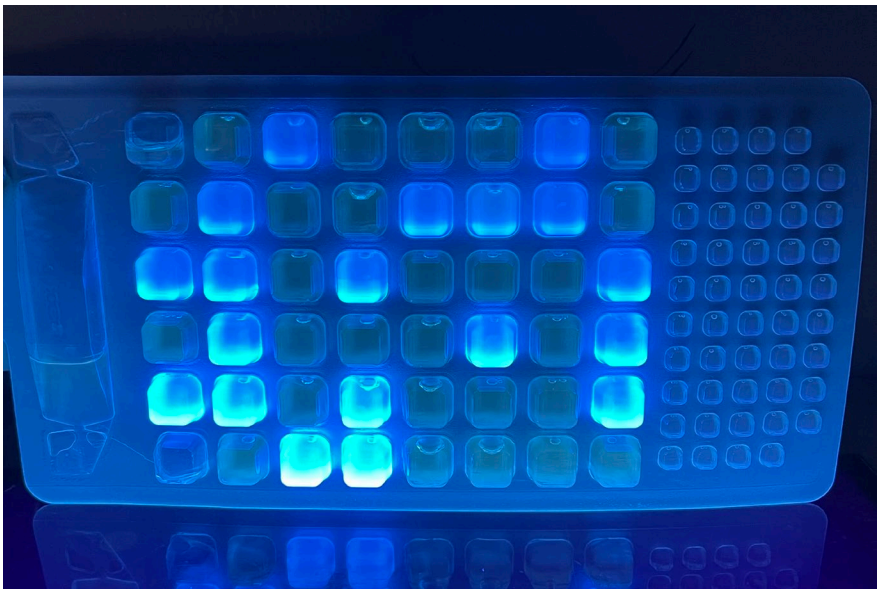

Figure 2. Image of Quanti-Tray® under UV light, showing fluorescence in enterococci-positive wells.

To perform the test, a 1:10 dilution is prepared by combining 10 mL of water sample with 90 mL of Milli-Q water and the Enterolert® reagent (Budnick et al., 1996). The mixture is then poured into 96-well IDEXX Quanti-Trays® (Quanti-Tray 2000® format), sealed, and incubated at 41°C 0.5°C for 24 hours (IDEXX Laboratories, Inc.). After incubation, the Quanti-Trays® are examined under UV light, with fluorescence indicating the presence of enterococci. The enterococci concentrations are then determined using an MPN reference table (Budnick et al., 1996).

While the Enterolert® Test is widely used due to its simplicity, affordability, and reliability, its 24-hour incubation period delays results, which may increase the risk of public exposure to contaminated water before health advisories can be issued. The EPA Method 1600, which uses membrane filtration, is another commonly used culture-based approach which also requires 24-hour incubation period. Molecular methods, such as the EPA Methods 1609 and 1611 can provide more rapid quantification of enterococci,

typically within a few hours. However, because enterococci concentrations are not indicative of fecal contamination in the tropics, there is a need for the use of microbial source tracking (MST) methods to identify the source of contamination (Fujioka et al., 2015). The use of human-associated markers such as CrAssphage and HF183, which target DNA from bacteria commonly found in human feces, is warranted for identifying sources of fecal contamination in the environment (Harwood et al., 2014). Targeting human contamination is a priority because it poses the greatest health risk, involving various enteric human pathogens, including highly specific human viruses such human noroviruses and enteroviruses. These markers can be detected using quantitative polymerase chain reaction (qPCR), which produces results in under two hours by directly amplifying genetic material (Wade et al., 2006; Converse, 2012). Although qPCR for enterococci or MST markers is time-efficient and offers greater sensitivity, its use is often limited by higher resource demands, including laboratory infrastructure, reagent costs and the need for replicates. Additionally, zoonotic pathogens remain a concern. As a result, culture-based methods remain the preferred approach in most monitoring programs, while qPCR based methods can provide additional information that is valuable.

1.4 ENTEROCOCCI: HUMAN VS. NON-FECAL SOURCES IN HAWAIʻI

Enterococci can survive and multiply under a wide range of environmental conditions, withstanding temperatures between 10°C and 45°C and pH levels from 4.8 to 9.6 (Torres et al., 2018). In tropical environments like Hawaiʻi, they tend to persist longer than other FIBs, such as *Escherichia coli,* in soils, marine waters, and moist beach sand (Torres et al., 2018). They can also remain viable for extended periods in sediments,

where they are more protected from environmental stressors compared to open water (Byappanahalli et al., 2012). Given Hawai‘i’s warm climate and environmental conditions, enterococci may multiply in natural environments, resulting in elevated concentrations in beach water, even in the absence of fecal contamination.

Additionally, Hawai‘i contains approximately 88,000 cesspools, with roughly 11,000 located on O‘ahu (Raine, 2019). Cesspools are deep, unlined ground pits that collect untreated human waste, posing a threat to groundwater, drinking water, streams, and marine environments (State of Hawaii, Department of Health Wastewater Branch, n.d.). In addition to cesspools, O‘ahu has nine wastewater treatment plants, located in Sand Island (Honolulu), Kahuku, Kailua, Lā’ie, Waimānalo, Honouliuli (‘Ewa Beach), Pa’ala a Kai (Waialua), Wahiawā, and Wai’anae (see Figure 3) (City and County of Honolulu, n.d.). During periods of heavy rainfall, untreated wastewater discharges and sewage spills become more frequent due to overflow events and limited treatment capacity (Rubinato et al., 2024). Such discharges can lead to elevated enterococci levels, creating unsafe conditions for recreational water users.

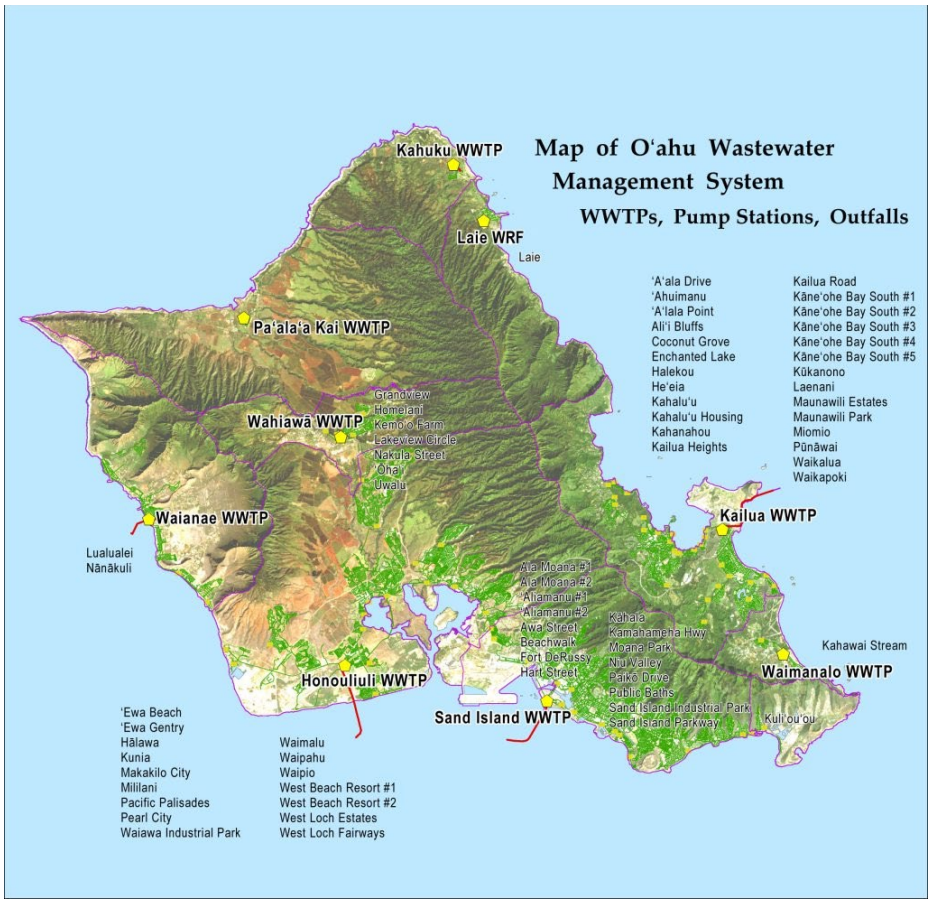

Figure 3. City and County of Honolulu Wastewater Treatment Plant locations (City and County of Honolulu, n.d.)

1.5 LIMITATIONS OF THE ENTEROLERT® TEST ACROSS CLIMATES

The specificity of the Enterolert® Test refers to its ability to accurately detect and differentiate enterococci from other bacteria present in water samples. In diagnostic terms, specificity is defined as the proportion of true negative results (Monaghan et al., 2021). When the test incorrectly identifies non-target species, it produces false positives, which lowers the test's specificity. The test's performance is often quantified by the rate of false positives (United States Environmental Protection Agency, 1985). Past studies have reported varying false-positive rates for the Enterolert® Test in different water types

and geographic locations. A study conducted in Connecticut, USA, testing both freshwater and marine recreational waters, found a false-positive rate of 5.1% (Budnick et al., 1996). Another study in Wellington, New Zealand, analyzing marine water samples, reported a false-positive rate of 2.4% (Abbott et al., 1998). In Southern California, USA, a study investigating marine and wastewater samples across multiple locations determined a false-positive rate of 8.4% (Ferguson et al., 2010). Several factors may contribute to variations in false-positive rates. The type of water tested, whether marine, freshwater, or drinking water, can influence enterococci survival, with their ability to withstand high salt concentrations and the origin of contamination affecting detection outcomes (Byappanahalli et al., 2012). Climate and water temperature also play a role, as environmental conditions impact bacterial persistence (see Section 1.4) (Torres et al., 2018). Additionally, microbial interference from non-enteric bacteria, such as *Bacillus licheniformis*, which shares some metabolic properties with enterococci, may contribute to false-positive readings (Peperzak et al., 2021).

2.0 METHODS

2.1 DETERMINATION OF THE ENTEROLERT® TEST SPECIFICITY

To assess the specificity of the Enterolert® Test, routine beach water quality monitoring samples were analyzed for enterococci and *Clostridium perfringens* (see Sections 1.1 and 1.2) between July 29, 2024, and November 12, 2024. A total of 49 enterococci-positive samples were used for this study. Each monitoring event included both positive and negative controls to ensure the accuracy of the test results. The positive control included *Enterococcus faecalis* BioBall® (bioMérieux), while the negative control used Milli-Q water. The Milli-Q water was generated from tap water using a Thermo Scientific Smart2Pure 6 UV/UF system which utilizes reverse osmosis, ultraviolet sterilization, and ultrafiltration (TEquipment, n.d.). Both controls were processed alongside routine samples to validate method performance. All positive controls produced expected fluorescence, and no fluorescence was observed in any of the negative controls across all 49 samples analyzed.

Following incubation, 49 Quanti-Trays® containing positive wells for enterococci were disinfected by wiping with ethyl alcohol to minimize contamination. The contents of 85 positive wells (Enterolert® isolates, n=85), were pipetted into sterile tubes, with many of the Quanti-Trays® containing multiple positive wells. These tubes were then frozen directly at -80 °C for later analysis. DNA was extracted and amplified following the Phusion® High-Fidelity DNA Polymerase kit, using the following reaction mixture for 50 µL reactions: 0.5 µL Phusion DNA Polymerase, 10 µL 5X Phusion HF buffer, 2.5 µL of 10 µM forward primer (27F), 2.5 µL of 10 µM reverse primer (1492R), 1µL of 10 mM dNTPs, and nuclease-free water to obtain a final volume of 50 µL. Primers are short

DNA sequences that initiate replication, with forward and reverse primers allowing DNA to be copied in both directions. A total of 5 µL of each Enterolert® isolate was combined with 45 µL of the master mixture, resulting in 50 µL per reaction. PCR amplification of the 16S rRNA gene fragment was performed using a thermal cycler (Bio-Rad CFX96™ Real-Time PCR System) under the following conditions: an initial denaturation at 98°C for 30 seconds, followed by 30 cycles of denaturation at 98°C for 10 seconds, annealing at 52°C for 20 seconds, extension at 72°C for 40 seconds, with a final extension at 72°C for 5 minutes before holding at 4°C for 5 minutes. Amplification is necessary to generate enough copies of DNA for detection, since DNA quantity in initial samples is often too low to directly analyze. Additionally, the 16S rRNA gene fragment is conserved across all bacteria, yet contains variable regions that allow for taxonomic differentiation.

PCR products were cleaned using Wizard® SV Gel and PCR Clean-Up System (Promega). The cleaning process involved adding 40 µL of membrane binding solution to each 50 µL amplified DNA sample, transferring the contents to a mini-column assembly, centrifugation at 16,000 x g for 1 minute. The flow-through was then discarded, and 700 µL of membrane wash solution (with ethanol added) was applied, followed by centrifugation at 16,000 x g for 1 minute. A second wash with 500 µL membrane wash solution was performed, followed by centrifuging at 16,000 x g for 5 minutes. Residual ethanol was evaporated through a final 1-minute centrifugation with the lid off, after which the mini-column was transferred to a new 1.5 mL microcentrifuge tube. Elution was performed by adding 50 µL of nuclease-free water, followed by centrifugation at 16,000 x g for 1 minute.

DNA quantification was performed using a Qubit fluorometer with the High Sensitivity (HS) Assay Kit. For each measurement, 190 µL of HS working solution was combined with 10 µL of each standard for calibration, while 195 µL HS working solution was combined with 5 µL of each purified DNA sample for quantification.

Each DNA sample was prepared for Sanger sequencing by setting up four separate sequencing reactions, each using a different primer: 27F, 515F, 981R, 1492R. Each sequencing reaction consisted of 5 µL of the cleaned PCR product, combined with 1 µL of a 3.2 µM primer, and was placed into an individual well of a 96-well plate. This allowed different regions of the 16S rRNA gene to be sequenced. The PCR plates was sent for sequencing to the ASGPB Sequencing Lab. Sequencing results were processed and analyzed using default settings in Geneious Prime (version 2025.0.3; Biomatters, Auckland, New Zealand). Sequences were aligned before species identification using BLAST. Geneious Prime provided the grade percentage, sequence length, percent identical sites, and the number of reads assembled. While most sequences included all four reads, some did not; in such cases, the software determined the most likely species based on the available sequence data.

To quantify the Enterolert® Test specificity, the false-positive rate was calculated by dividing the number of non-*Enterococcus* species by the total number of Enterolert® isolates (n=85), expressed as a percentage. This analysis provided insight into the accuracy of the Enterolert® Test in differentiating enterococci from non-*Enterococcus* species in beach water samples in Hawai‘i.

2.2 MICROBIAL SOURCE TRACKING

Water samples received from the HDOH were refrigerated at 4°C following initial water quality monitoring to preserve microbial integrity. After 24 hours, enterococci-positive water samples (~300 mL) were selected for further analysis. To retain the viral fraction, the samples were acidified to pH 3.5 using hydrochloric acid (HCl) and filtered through 0.45 µm pore-size membrane filters using a vacuum filtration system. Filters were then removed using sterile forceps, placed in ZR BashingBead™ Lysis Tubes, and stored at -80°C until DNA extraction.

DNA was extracted using the Quick-DNA™ Fecal/Soil Microbe Microprep Kit (Zymo Research), following the manufacturer's protocol. 750 µL of BashingBead™ Buffer was added to each BashingBead™ Lysis Tube containing the filters. The tubes were processed in a Mini Beadbeater™ MBB-8 (BioSpec Products) for two rounds of bead beating, each lasting two minutes, to ensure cell disruption. Each tube was then centrifuged at 10,000 x g for 1 minute, followed by transferring 400 µL of the supernatant to a Zymo-Spin™ III-F Filters in collection tubes and centrifugation at 8,000 x g for 1 minute. The Zymo-Spin™ III-F Filters were discarded and 800 µL of Genomic Lysis Buffer and 400 µL of 95% ethanol were added to each collection tube. 800 µL of this mixture was then added to Zymo-Spin™ IC Columns in collection tubes and centrifuged at 10,000 x g for 1 minute. Any flow-through was discarded and the previous step was repeated. 200 µL of DNA Pre-Wash Buffer was then added to Zymo-Spin™ IC Columns in new collection tubes and centrifuged at 10,000 x g for 1 minute. 500 µL g-DNA Wash Buffer was added to the Zymo-Spin™ IC Columns and centrifuged at 10,000 x g for 1 minute. The Zymo-Spin™ IC Columns were then added to clean 1.5 mL

microcentrifuge tubes and 50 µL DNA Elution Buffer was added to the column matrixes and centrifuged at 10,000 x g for 30 seconds to elute the DNA. Zymo-Spin™ II-µHRC Filters were then placed in new collection tubes and 600 µL of Prep Solution was added, followed by centrifugation at 8,000 x g for 3 minutes. The eluted DNA was then transferred to these prepared Zymo-Spin™ II-µHRC Filters in clean 1.5 mL microcentrifuge tubes and centrifuged at 16,000 x g for 3 minutes

Quantitative PCR (qPCR) was used to detect the presence of human-associated fecal contamination markers, specifically CrAssphage and HF183. The GoTaq® qPCR Master Mix (Promega) was used with each 20 µL reaction containing 10 µL of GoTaq® qPCR Master Mix, 1 µL of 10 µM forward primer, 1 µL of 10 µM reverse primer, 0.3 µL of probe, 2.7 µL of nuclease-free water, and 5 µL DNA sample. A 10-fold serial dilution of standards was prepared ranging from 3.0×10^6 gene copies (gC) to 3.0×10^0 gene copies per reaction. Three replicates of each standard concentration were included, and each unknown DNA sample was run in duplicate to ensure consistency across reactions. Additionally, negative controls were included with three replicates containing nuclease-free water, as well as positive control wastewater samples from Sand Island, Honouliuli and Hawai‘i Kai wastewater treatment plants. PCR plates were sealed and centrifuged to ensure efficient reagent distribution, then thermal cycled with an initial denaturation at 95°C for 3 minutes, followed by 40 cycles of denaturation at 95°C for 10 seconds and annealing/extension at 60°C for 30 seconds. Amplification data were then assessed using Bio-Rad CFX Manager software.

3.0 RESULTS

3.1 THE ENTEROLERT® TEST SPECIFICITY

3.1.1 BEACH WATER SAMPLE LOCATION

A total of 176 beach water samples were collected from 24 coastal locations routinely monitored by the HDOH between July 29, 2024 and November 12, 2024. Only beaches that presented at least one enterococci-positive sample during this time frame were included in this analysis, therefore beaches that were consistently negative for enterococci were not represented in this count. Of the included samples, 49 (27.8%) tested positive for enterococci, however none exceeded the BAV of >130 MPN/100 mL. All enterococci concentrations ranged from 10 to 84 MPN/100 mL, indicating overall good water quality during the sampling period. Additionally, 15 of the 24 beaches were classified as Tier 1, 6 as Tier 2, and 3 were assumed to be Tier 3 or higher, based on the absence of classification information on the HDOH website.

Ala Moana Beach Park contributed the highest number of enterococci-positive samples (n=10). This location includes multiple sampling sites: Ala Moana Park, Center Pt. 2; Ala Moana Park, D.H Pt. 2; Ala Moana, Ewa; and Ala Moana Lagoon, which were combined into a single location for visualization (Figure 4). Similarly, Kapiʻolani Park and Kapiʻolani Park Pt. 2 were grouped together. Other beaches with a relatively high number of positive samples included Lanikai Beach shoreline, Moana Beach, and Kuhio Beach (Waikīkī Pt. 2), each with 4 enterococci-positive samples. As expected, these beaches are classified as Tier 1 monitoring sites (Table 1 and 2) and are therefore sampled more frequently. However, raw counts of positive samples alone do not fully reflect the relative contamination risk. When normalized by the total number of samples

collected per beach (including both positive and negative results), several other sites exhibited higher enterococci detection rates. For instance, Kapaeloa had 2 positive results out of 3 samples (66.7%), Kawaikui Beach Park had 3 out of 5 (60%), Kapi‘olani and Kailua Beach Park each had 3 out of 7 (42.9%) and Lanikai Beach Shoreline had 4 out of 10 (40%). This highlights the importance of considering sampling frequency when interpreting detection patterns. Although Tier 1 beaches contributed more enterococci-positive samples, their normalized detection rates were often lower than some sites that were sampled less frequently. Importantly, despite repeated detections, no exceedances of the BAV were observed at any sites, emphasizing that detection frequency alone does not equate to poor water quality.

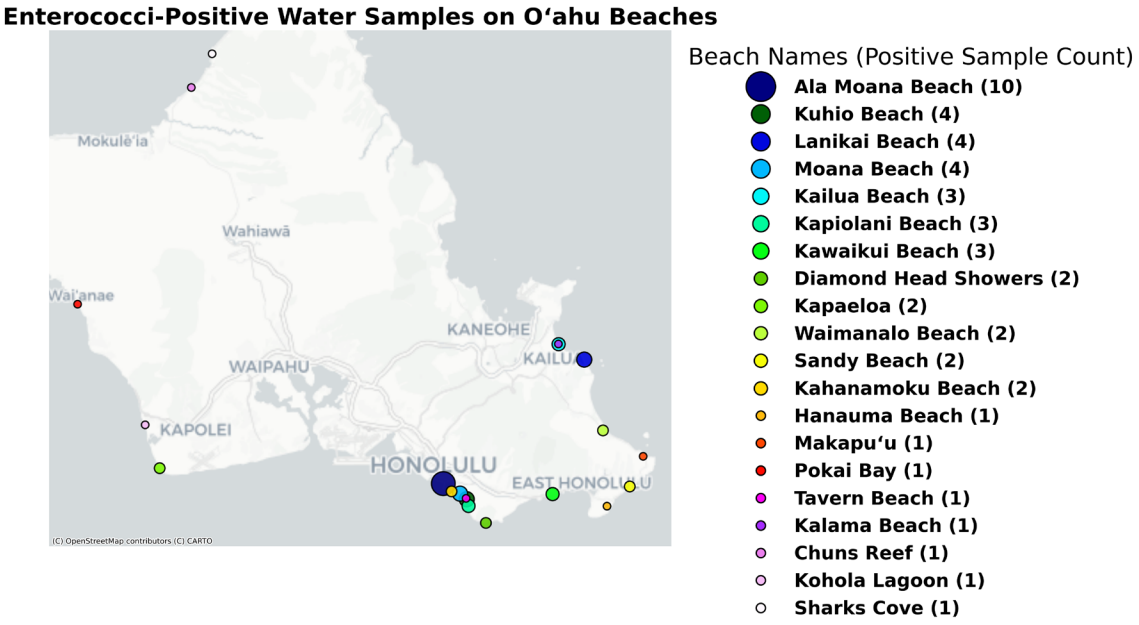

Figure 4. Locations of enterococci-positive samples (n=49), with dot size indicating the number of positive detections per site. Beaches classified as Tier 1 and Tier 2 monitoring sites (see Table 1) were sampled more frequently, which explains the higher number of detections observed at these locations.

Beach ID	Beach Name	Tier (1, 2, 3+)	Positive Samples	Positive Wells
HI681782	Kuhio Beach, Waikiki Pt. 2	1	4	11
HI596989	Lanikai Beach Shoreline	1	4	11
HI898947	Moana Beach Park	1	4	6
HI882094	Ala Moana Park, Center Pt.2	1	3	3
HI306071	Ala Moana Park, D.H Pt.2	1	3	6
HI482719	Kailua Beach Park Pt.2	1	3	6
HI776760	Sandy Beach Pt.1	1	2	2
HI366432	Kahanamoku Beach, Waikiki Pt.2	1	2	2
HI471097	Waimānalo beach pt.2	1	2	6
HI451471	Hanauma Beach Park	1	1	1
HI723399	Makapu‘u	1	1	1
HI659533	Poka’i Bay	1	1	1
HI950962	Chuns Reef	1	1	1
HI515191	Kohola Lagoon I	1	1	2
HI19395	Shark’s Cove (Snorkeling Spot)	1	1	1

Table 2.1. Enterococci-positive samples categorized as Tier 1

HI304424	Kawaikui Beach Park	2	3	4
HI702973	Ala Moana, Ewa	2	3	3
HI904851	Kapaeloa	2	2	2
HI733929	Kapi‘olani Park	2	2	2
HI733929	Kapi‘olani Park Pt. 2	2	1	1
HI071892	Kalama Beach	2	1	3
NA	Diamond Head Showers	3+	2	6
NA	Tavern Beach	3+	1	2
NA	Ala Moana Lagoon	3+	1	2
Total	24	Tier 1: 15 Tier 2: 6 Tier 3+: 3	49	85

Table 2.2. Enterococci-positive sample categorized as Tier 2 and 3+

Beaches are categorized by HDOH monitoring tier (see Table 1), which reflects the priority and frequency of routine water quality monitoring sampling. Tier 1 beaches are sampled most frequently, followed by Tier 2, then Tier 3 or higher. Tables 2.1 and 2.2 include HDOH-assigned beach IDs, the number of enterococci-positive samples, and the number of positive wells (as determined from Quanti-Trays®) recorded at each beach site.

3.1.2 SPECIES IDENTIFICATION AND ENTEROLERT® TEST SPECIFICITY

A total of 85 enterococci-positive wells (Enterolert® isolates) were analyzed to determine the specificity of the Enterolert® Test. Results indicate a false-positive rate of 11.76%, with 10 out of 85 wells containing non-E*nterococcus* species, suggesting that the Enterolert® Test is susceptible to detecting non-target bacteria. Among the non-E*nterococcus* species identified, *Vagococcus luciliae* (Ala Moana Park Center, Pt. 2, Chuns Reef, Shark's Cove (Snorkeling Spot)) was the most frequently detected, found in 3 wells (3.53%). This was followed by *Clostridium tertium* (Kawaikui Beach Park, Kailua Beach Park Pt. 2) identified in 2 wells (2.35%). Additionally, five other species were detected in single wells (1.18%), including *Streptococcus gordonii* (Ala Moana Park D.H Pt. 2), *Bacillus* (Ala Moana, Ewa), *Vagococcus bubulae* (Kapaeloa), *Paenibacillus dendritiformis* (Ala Moana, Ewa), and *Vagococcus fluvialis* (Ala Moana Park, Center Pt. 2). These non-target species emphasize potential limitations in the Enterolert® Test's specificity, possibly influenced by Hawai'i's tropical coastal environment. Despite these false-positives, 88.24% of wells (75 out of 85) were confirmed to contain *Enterococcus* species, supporting the conclusion that the Enterolert® Test is a generally effective tool for detecting enterococci in beach water samples. Among *Enterococcus*-positive wells, *E. faecalis* (30.5%) and *E. faecium* (29.4%) were the dominant species. These species are commonly associated with human fecal waste and can serve as indicators of potential public health risks. The remaining 40.1% of *Enterococcu*s detections consisted of species including *E. mundtii* (4 wells), *E. casseliflavus* (4 wells), *E. gallinarum* (3 wells), *E. hirae* (3 wells), *E. innesii* (1 well), *E. raffinosus* (1 well), *E. gilvus* (1 well), *E. avium* (1 well), *E. asini* (1 well), and *E.*

thailandicus (1 well). Additionally, several wells contained multiple species, indicating mixed bacterial populations in these samples. These combinations included *E. casseliflavus* and *E. gallinarum* (3 wells), *E. faecium* and *E. Lactic* (1 well), *E. raffinosus* and *E. gilvus* (1 well), and *E. innesii* and *E. gallinarum (*1 well). The occurrence of mixed *Enterococcu*s species within single wells, emphasizes the need for molecular confirmation to support culture-based methods for detecting enterococci in water samples. Overall, while the Enterolert® Test proved to be generally accurate, the detection of non-*Enterococcus* species suggests that positive results may require verification—particularly in tropical environments such as Hawai‘i, where non-enteric bacteria may share similar metabolic pathways and environmental conditions naturally support the survival of enterococci. This considerably higher false-positive rate contrasts with findings from past studies conducted in temperate regions (see Sections 1.5 and 4.1) and underscores the importance of considering regional environmental factors when interpreting the Enterolert® Test results.

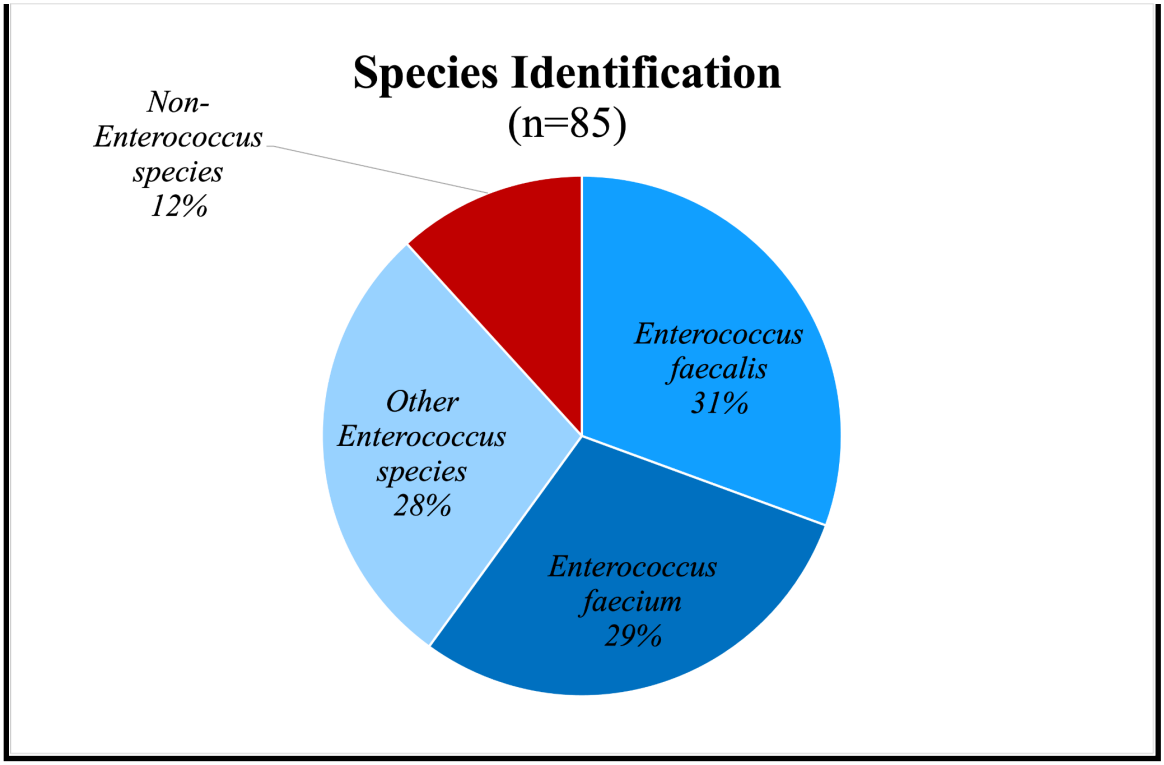

Figure 5. Species identification of Enterolert® positive wells (n=85), showing the proportion of *Enterococcus faecalis, Enterococcus faecium*, other *Enterococcus* species, and non-*Enterococcus* species (false positives) detected across all water samples.

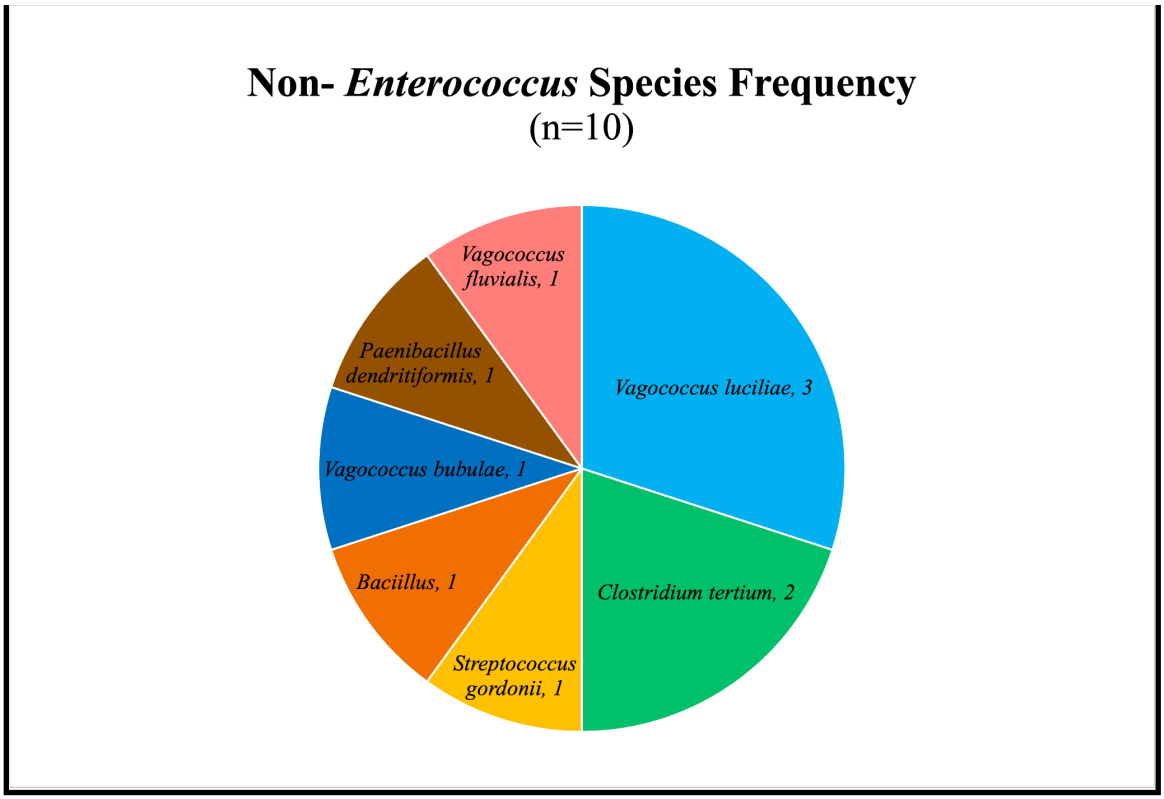

Figure 6. Non-*Enterococcus* species identified among false-positive wells (n=10).

3.2 HUMAN FECAL CONTAMINATION DETECTION

3.2.1 CRASSPHAGE AND HF183 PRESENCE PER BEACH

Human-associated microbial source tracking markers—CrAssphage and HF183—were detected in 31 of the 49 enterococci-positive samples (63.3%), and were present at 17 of the 24 beaches sampled (70.8%). MST was only performed on enterococci-positive samples, so it is assumed that the 127 samples that were enterococci-free did not have significant sewage contamination. CrAssphage was present in 21 samples (42.9%) and HF183 in 18 samples (36.7%) (see Table 3). 18 samples (36.7%) had no detectable human markers, indicating contamination from non-human sources, such as the environment. Eight samples (16.3%) contained both markers, providing strong evidence

of fecal pollution. These dual detections occurred at five beaches: Lanikai Beach Shoreline (1 sample), Ala Moana Beach Park (3 samples), Moana Beach (2 samples), Kuhio Beach (1 sample), and Sandy Beach (1 sample), allowing for double confirmation of fecal contamination.

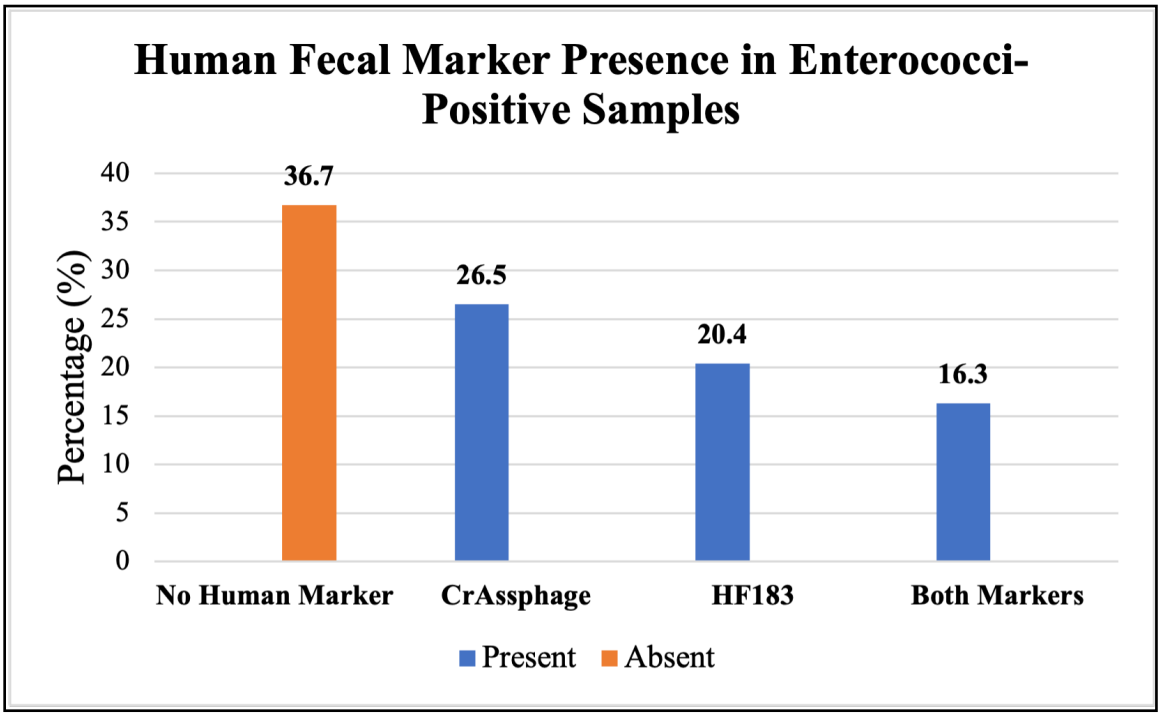

Figure 7. Presence of human-associated fecal markers (CrAssphage and HF183) in Enterococci-positive beach water samples

At the beach level, Ala Moana Beach Park, showed the highest number of human marker detections, with 7 samples positive for CrAssphage (70%) and 5 for HF183 (50%) out of 10 total enterococci-positive samples. Moana Beach followed with 4 CrAssphage detections (100%), and 2 for HF183 detections (50%) out of 4 positive samples. Other beaches with multiple detections included Kuhio Beach, Kailua Beach Park, Lanikai Beach Shoreline, and Kapiʻolani Park. Beaches where only one marker was detected in a single sample included Kahanamoku Beach, Sandy Beach, Hanauma Bay, Makapuʻu,

Kohola Lagoon I, Pōka‘ī Bay, and Tavern Beach. In contrast, seven beaches had no detectable human fecal markers: Chuns Reef, Shark’s Cove, Kapaeloa, Waimānalo Beach, Kalama Beach, Kawaikui Beach and the Diamond Head Beach showers. These locational trends highlight a pattern in which human marker detections were more common on O ahu’s southern beaches, particularly in more urbanized areas such as Honolulu. These areas are likely subject to higher population density, increased urban runoff, and aging wastewater infrastructure, contributing to elevated detection rates.

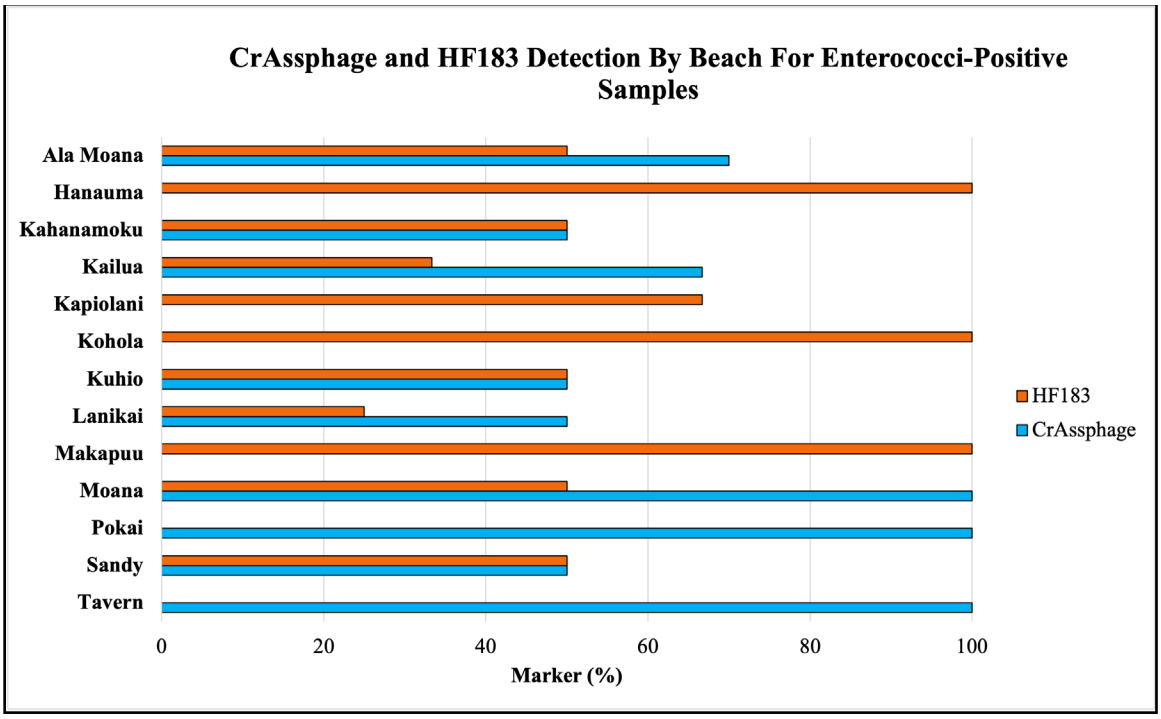

Figure 8. CrAssphage and HF183 detection by beach location. Bars represent the percentage of human fecal markers present in enterococci-positive samples per beach location (see Table 3).

FREQUENCY OF CRASSPHAGE AND HF183 MARKERS IN ENTEROCOCCI-POSITIVE SAMPLES AT EACH BEACH LOCATION

Beach Location	Total Samples Collected	No. of Positive Samples	CrAssphage Detection (%)	HF183 Detection (%)
Waimanalo Beach	11	2 (18.2%)	0 (0%)	0 (0%)
Kawaikui Beach Park	5	3 (60%)	0 (0%)	0 (0%)
Lanikai Beach Shoreline	10	4 (40%)	2 (50%)	1 (25%)
Kalama Beach	4	1 (25%)	0 (0%)	0 (0%)
Kahanamoku Beach, Waikiki	9	2 (22.2%)	1 (50%)	1 (50%)
Moana Beach Park	11	4 (36.4%)	4 (100%)	2 (50%)
Sandy Beach	11	2 (18.2%)	1 (50%)	1 (50%)
Hanauma Beach Park	11	1 (9.1%)	0 (0%)	1 (100%)
Ala Moana Beach Park	38	10 (26.3)	7 (70%)	5 (50%)
Kailua Beach Park	7	3 (42.9%)	2 (66.7%)	1 (33.3%)
Kuhio Beach, Waikiki	13	4 (30.8%)	2 (50%)	2 (50%)
Makapuu	11	1 (9.1%)	0 (0%)	1 (100%)
Kapaeloa	3	2 (66.7%)	0 (0%)	0 (0%)
Pokai Bay	3	1 (33.3%)	1 (100%)	0 (0%)
Tavern Beach	6	1 (16.7%)	1 (100%)	0 (0%)
Diamond Head Beach Showers	6	2 (33.3%)	0 (0%)	0 (0%)
Kapiolani Park	7	3 (42.9%)	0 (0%)	2 (66.7%)
Chuns Reef	4	1 (25%)	0 (0%)	0 (0%)
Kohola Lagoon I	3	1 33.3%)	0 (0%)	1 (100%)
Shark's Cove	3	1 (33.3%)	0 (0%)	0 (0%)
Total	176	49 (27.8%)	21 (42.9%)	18 (36.7%)

Table 3. Frequency of CrAssphage and HF183 markers in enterococci-positive samples at beach location.

To further explore the relationship between enterococci and human-associated fecal contamination, correlations were assessed between the number of enterococci-positive samples and the number of samples positive for CrAssphage and HF183 per beach. As shown in Figure 9, strong positive association was found between enterococci and CrAssphage ($r = 0.89$, $p = 1e–07$) and HF183 ($r = 0.87$, $p = 5.3e–07$), indicating that beaches with more enterococci-positive samples also tended to have more detections of human-associated markers. These findings suggest that while enterococci alone may not reliably distinguish contamination sources and identify health risk, their presence may

reflect an increased likelihood of human fecal pollution.

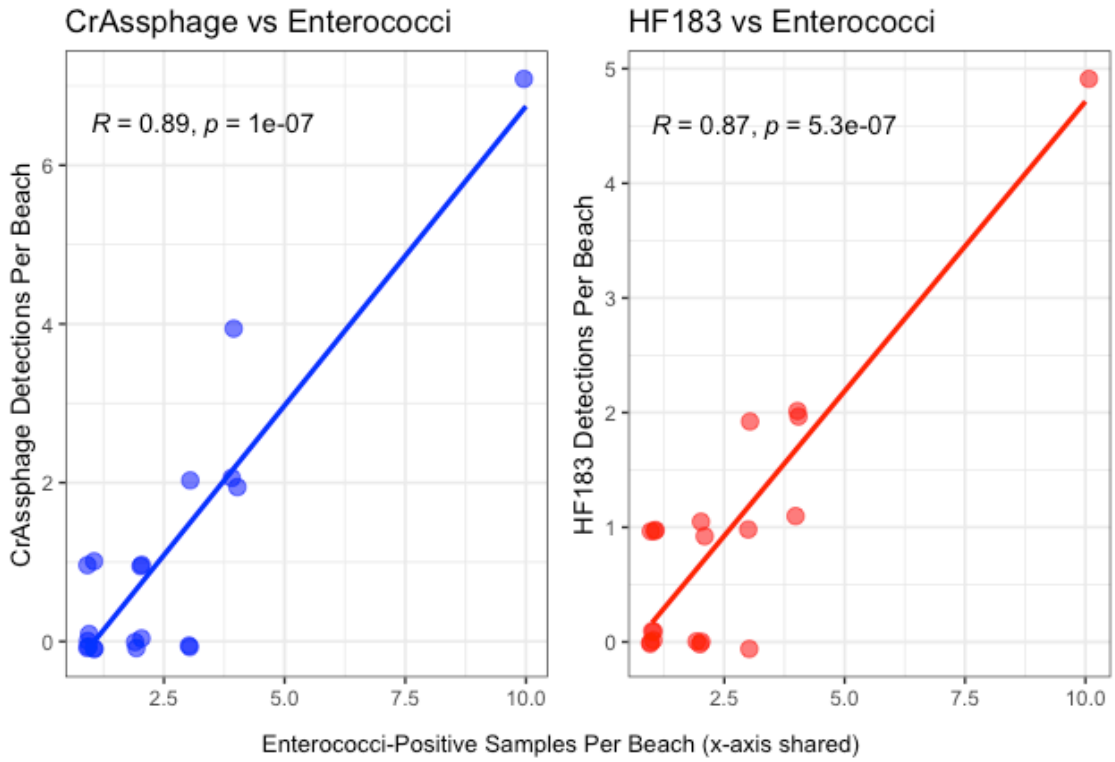

Figure 9. Scatterplots showing the relationship between the number of enterococci-positive samples and the number of human-associated fecal marker detections per beach (CrAssphage on the left, HF183 on the right). Each point represents a single beach. Strong positive correlations were observed between enterococci and CrAssphage (R = 0.89, p = 1e–07) and HF183 (R = 0.87, p = 5.3e–07) at the beach level.

3.2.2 CRASSPHAGE AND HF183 CONCENTRATIONS

Overall, the geometric mean $\log_{10}$ concentrations of CrAssphage and HF183 were approximately 2.5 and 2.2 $\log_{10}$ gene copies per 100 mL, respectively. This corresponds to roughly 570 gC/100 mL and 180 gC/100 mL when considering raw concentrations. CrAssphage showed more variability in concentrations compared to HF183 as indicated

by a wider interquartile range (IQR) (Figure 10). Given the raw concentrations, CrAssphage concentrations ranged from approximately 56 gC/100 mL to 2,000 gC/100 mL, while HF183 concentrations ranged from 70 gC/100 mL and 300 gC/100 mL. The median concentration for CrAssphage and HF183 were 240 gC/100 mL and 150 gC/100 mL, however the mean concentration were very distinct, with 570 gC/ mL and 180 gC/100 mL. Given the higher variability of CrAssphage concentrations, this marker may be more sensitive for detecting human fecal pollution when compared to HF183. Additionally, the controls which included wastewater samples from Sand Island, Honouliuli, and Hawai‘i Kai wastewater treatment plants had a mean concentration of roughly 6.4 $\log_{10}$ gene copies per 100 mL. This highlights that although these human-associated markers were detected in Oahu beach water samples, their concentrations were significantly lower than those observed in raw sewage, suggesting that the contamination present was likely at trace levels. Furthermore, the concentrations of human-associated markers typically exceed enterococci concentrations 10-100 fold in sewage, hence the marker can be detected in environmental waters even when enterococci concentrations have decreased below detectable levels (Kirs, 2021). Given the sensitivity of these markers due to the elevated concentrations in raw sewage, their presence is not necessarily indicative of a significant human health risk.

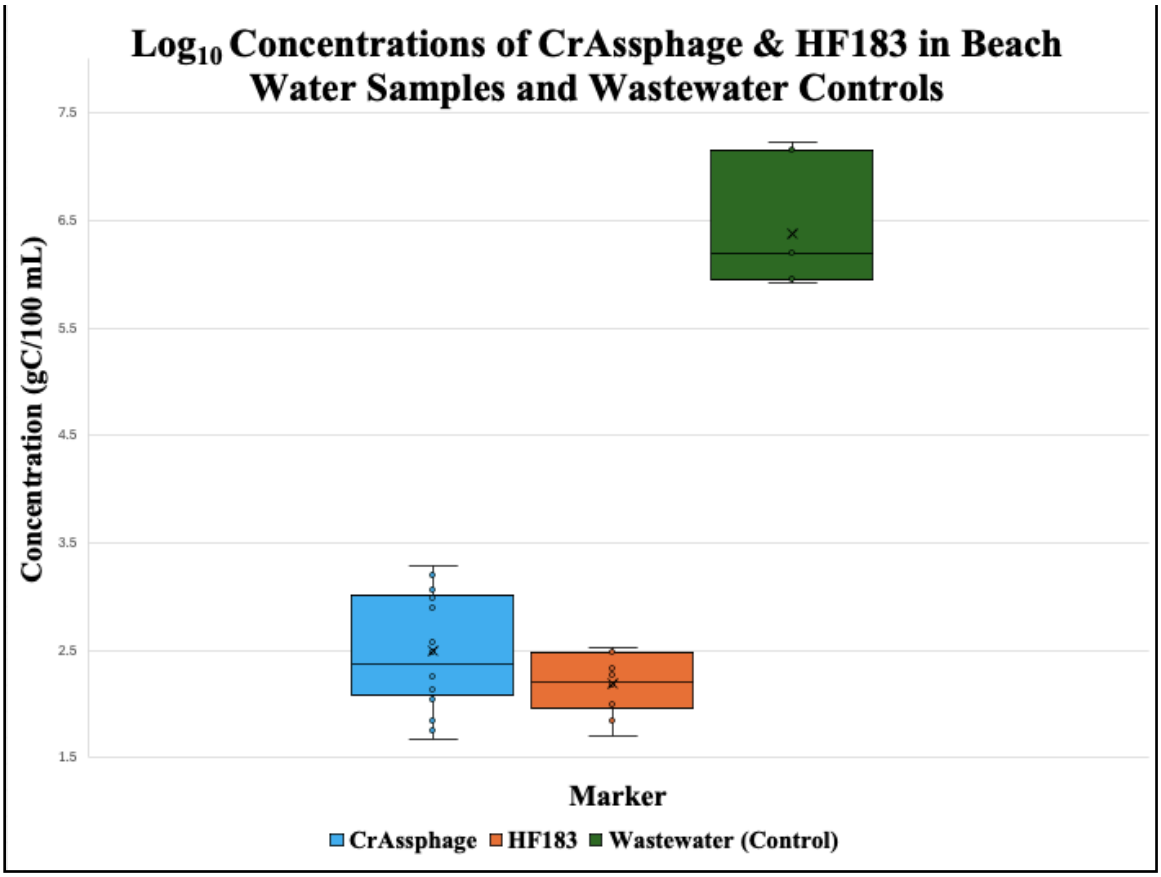

Figure 10. Log_{10} transformed concentrations of CrAssphage and HF183 (gc/100 mL) in beach water samples and wastewater controls. Boxes represent the interquartile range (IQR), with the interior horizontal line indicating the median concentration. Whiskers extend to1.5 x IQR, and mean concentrations are shown by "X" within each box.

3.2.3 PROXIMITY TO WWTPS

The presence of enterococci-positive samples and human-associated fecal markers (CrAssphage and HF183) may be influenced by the proximity of beach location to nearby wastewater treatment plants. Of the 24 beaches from which samples were collected, one beach was located within 1 km of a WWTP, three were within 3 km, and the remaining beaches were within 10 km (see Figure 3 and 4). Among the four beaches located within

1-3 km, three had at least one sample with detectable human markers. At Pōkaʻī Bay (located just under 1 km from the Waiʻanae WWTP), one sample (100%) tested positive for CrAssphage. Kailua Beach—approximately 3 km from Kailua WWTP— had two samples positive for CrAssphage (66.7%) and one positive for HF183 (33.3%). Makapuʻu Beach, about a km from the Waimānalo WWTP, had one HF183 positive sample (100%). Several beaches in urban Honolulu, roughly 4 to 7 km from the Sand Island WWTP, showed the highest number of human-associated marker detections. Ala Moana Beach had 7 samples positive for CrAssphage (70%) and 5 for HF183 (50%); Moana Beach had 4 and 2 positive samples respectively (100%, 50%); and Kuhio Beach had 2 samples positive for both (50% each). Additional detections occurred at Kapiʻolani Beach (2 HF183, 66.7%), Kahanamoku (1 CrAssphage, 50% and 1 HF183, 50%), and Tavern Beach (1 CrAssphage, 100%). These findings suggest that proximity to WWTPs may contribute to fecal contamination in coastal waters, particularly in urban regions. However, the presence of human-fecal markers was not confined to sites near wastewater treatment plants, suggesting that others sources such as cesspools, stormwater runoff or leaking infrastructure may also contribute to fecal contamination. This further highlights the importance of the Tier Monitoring System developed by the HDOH (see Table 1), which ensures that high-risk beaches are sampled more frequently. Additionally, incorporating MST tools into this framework can substantially improve the ability to detect and attempt to characterize fecal pollution, particularly in high-risk areas.

3.2.4 FALSE-POSITIVE SPECIES AND HUMAN MARKER OVERLAP

Of the ten false-positive wells (wells containing non-*Enterococcus* species; see Sections 3.1.2), half of these (5/10) had at least one human-associated fecal marker (CrAssphage or HF183) present. Notably, nearly all false-positive samples associated with human markers (4 of 5) were found at Ala Moana Beach Park, including detections of *Streptococcus gordonii, Bacillus, and Vagococcus luciliae, Vagococcus fluvialis*. Additionally, at Kailua Beach Park, where *Clostridium tertium* was identified, HF183 was present concurrently. This pattern suggests that many of the false-positives, particularly at Ala Moana Beach Park, may not be false in terms of public health relevance. Although these wells contained non-*Enterococcus* species, the concurrent detection of human fecal markers indicates true fecal contamination. Therefore, while the Enterolert® Test may overestimate enterococci presence due to non-target detections, it did not entirely overestimate the underlying risk. Conversely, at beaches where false positives were detected and no human markers were present—such as Kawaikui, Kapaeloa, Chuns Reef, and Shark's Cove—the results suggests that environmental interference was the more likely source of enterococci. This underscores the importance of interpreting Enterolert® Test results alongside molecular data in tropical regions like Hawai'i, where false-positive readings may still signal microbial contamination. Although the Enterolert® Test was found to have a false-positive rate of nearly 12%, it appears to still be useful in identifying non-*Enterococcus* bacteria that may be associated with fecal pollution.

False-Positive Species and Human Marker Detection by Beach

Beach Location	False-Positive Species	CrAssphage Presence	HF183 Presence
Ala Moana Beach Park	*Streptococcus gordonii*	Yes	Yes
Ala Moana Beach Park	*Bacillus*	Yes	Yes
Ala Moana Beach Park	*Vagococcus luciliae*	No	Yes
Ala Moana Beach Park	*Vagococcus fluvialis*	Yes	No
Ala Moana Beach Park	*Paenibacillus dendritiformi*	No	No
Kailua Beach Park	*Clostridium tertium*	No	Yes
Kawaikui Beach Park	*Clostridium tertium*	No	No
Kapeloa	*Vagococcus bubulae*	No	No
Chuns Reef	*Vagococcus luciliae*	No	No
Shark's Cove	*Vagococcus luciliae*	No	No
Total	10	3	4

Table 4. False-positive bacterial species identified and corresponding detection of human fecal markers (CrAssphage and HF183). "Yes" indicates marker presence in the sample containing the specified species. Species are organized by beach location; all species are non-*Enterococcus.*

3.3 ADDITIONAL INDICATORS OF BEACH WATER QUALITY

3.3.1 ENTEROCOCCI LEVELS VS. *CLOSTRIDIUM PERFRINGENS*

Analysis of the 49 beach water samples used in this study revealed no correlation between enterococci and *Clostridium perfringens* levels. The calculated R^2 value of 0.0235 indicates that only 2.35% of the variability in enterococci concentrations can be explained by the levels of *C. perfringens* present in these samples. This very low R^2 value suggests that *C. perfringens* is not an effective explanatory variable for predicting enterococci concentrations in Hawai‘i beach water. Although both are used as fecal

indicator bacteria to assess water quality, these findings demonstrate that *C. perfringens* does not reliably signal the presence or absence of enterococci, implying that they may originate from different sources or respond differently to environmental conditions. Additionally, none of the 49 samples exceeded the State of Hawai‘i’s recommended *C. perfringens* threshold of <50 CFU/100 mL in marine water (State of Hawaii, 2012).

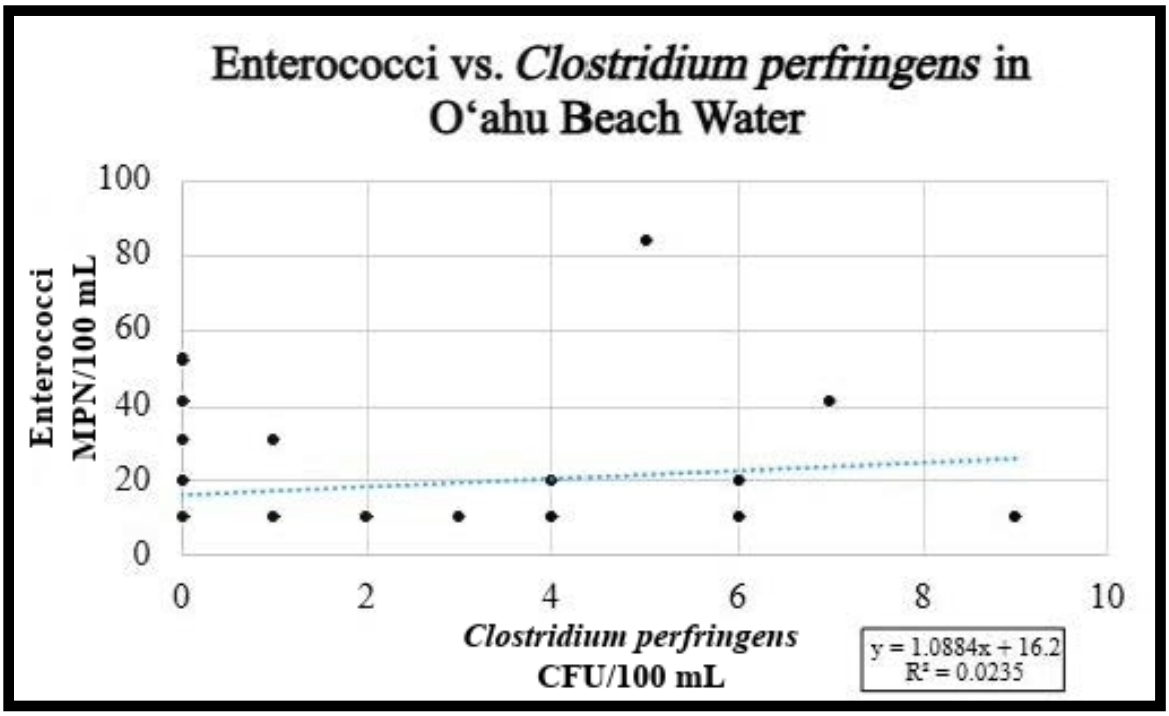

Figure 11. Relationship between Enterococci and *Clostridium perfringens* concentrations in beach water samples. Note: Values labeled as 0 represent measurements below the detection limit (<1).

4.0 DISCUSSION

4.1 LIMITATIONS OF ENTEROCOCCI AS A FIB IN HAWAI'I

While enterococci continue to serve as a primary indicator used to determine water quality in Hawai'i, this study reveals the limitations of the Enterolert® Test in tropical environments. Among the 176 samples collected, 27.8% tested positive for enterococci, yet none exceeded the regulatory threshold of 130 MPN/100 mL—likely due to sampling occurring during Hawai'i's dry season, when exceedances are less common (Price, 1983). Given that species identification revealed a false-positive rate of 11.76% —notably higher than previously reported rates in temperate regions (see section 1.5)—it appears that the Enterolert® Test is prone to detecting non-*Enterococcus* bacteria in Hawai'i's tropical setting. Such findings challenge the test's specificity in environments where enterococci may persist or multiply in non-fecal sources like beach sand, soil and sediments (see Section1.4) This affirms that enterococci may not be a suitable FIB to indicate true fecal contamination in tropical regions.

The detection of human fecal markers in a large portion of the enterococci-positive samples (63.3%) confirms the presence of sewage contamination in Hawai'i beach water, raising important public health concerns. Although detected at low concentrations, the presence of human fecal markers still signals potential public health risks, as it suggests the possible occurrence of other harmful pathogens. These findings point to a need for improved wastewater infrastructure and stormwater management, particularly near beaches that are regularly used recreationally. Additionally, 36.7% of the samples lacked human markers, further reinforcing that enterococci can originate

from non-fecal sources. This highlights the limited ability of enterococci to distinguish between environmental and fecal contamination in Hawai'i beach waters.

However, the Enterolert® Test's value as a preliminary screening tool should still be noted. 50% of wells identified as false positives (non-*Enterococcus* species) still contained CrAssphage or HF183, pointing to human fecal contamination even when *Enterococcus* species were not present. This was most evident at Ala Moana Beach Park, where urban runoff and proximity to Sand Island WWTP may have contributed to contamination. This indicates that even when the Enterolert® Test detects non-target species, it may still capture samples impacted by human fecal contamination. Therefore, despite its limitations it can still be useful in signaling potential health risks. However, its tendency to overestimate contamination still demonstrates that enterococci are not a fully reliable FIB, which may lead to unnecessary public health advisories or beach closures triggered by non-*Enterococcus*, environmental sources.

These findings support that the integration of MST into routine beach water quality monitoring would improve the accuracy of results. Unlike culture-based methods, MST tools such as CrAssphage and HF183 allow for direct detection of human fecal contamination offering greater specificity than the Enterolert® Test. This would allow for better differentiation between environmentally persistent enterococci and sewage-related contamination, providing more reliable public health risk assessments, especially for tropical regions like Hawai'i where environmental conditions challenge the assumptions behind the water quality standards of temperate geographic locations.

5.0 CONCLUSION

This study highlights the need to reevaluate the sole reliance on enterococci as a fecal indicator bacteria in Hawai i. In tropical environments, where conditions allow enterococci to naturalize and grow in extra-enteric habitats such as soils and sediments, enterococci may not be reliable indicators due to these natural reservoirs. The observed higher false-positive rates of the Enterolert® Test in Hawai i, compared to temperate regions, suggests that this method may overestimate enterococci concentration in tropical settings. These limitations hinder our ability to make well informed and protective public health decisions.

To better align water quality monitoring with actual health risks, secondary indicators and MST tools should supplement the results of traditional methods such as the Enterolert® Test, allowing for more informed beach management strategies. Additionally, improvements to wastewater and stormwater systems are essential, especially in urban regions of O ahu, which could certainly improve the water quality. Ultimately, refining monitoring methods is critical for evaluating water quality in Hawai i, to ensure clean and safe coastal waters.

APPENDIX: SUPPORTING INFORMATION

CODE LISTING

LIST OF FIGURES

Code S1: Python Script for Enterococci-Positive Water Samples on O ahu Beaches

Map (Figure 4) Map. Geopandas, Contextily and Matplotlib were used to plot locations based on latitude and longitude and scales each point relative to the number of enterococci-positive samples. A Carto basemap (from Contextily) was utilized, providing geographical context.

```
import geopandas as gpd
import pandas as pd
import matplotlib.pyplot as plt
import contextily as ctx
import numpy as np

#Enterococci-Positive Water Samples at O‘ahu Beaches
data = {
    'Beach': ['Waimanalo Beach', 'Kawaikui Beach', 'Lanikai Beach',
'Kalama Beach', 'Kahanamoku Beach', 'Moana Beach', 'Sandy Beach',
'Hanauma Beach', 'Ala Moana Beach', 'Kailua Beach', 'Kuhio Beach',
'Makapu‘u', 'Kapaeloa', 'Pokai Bay', 'Tavern Beach', 'Diamond Head
Showers', 'Kapiolani Beach', 'Chuns Reef', 'Kohola Lagoon', 'Sharks
Cove'],
    'Latitude': [21.3330, 21.2793, 21.3925, 21.4058, 21.2815, 21.2797,
21.2857, 21.2690, 21.2882, 21.4056, 21.2750, 21.3111, 21.3012, 21.4393,
21.2757, 21.2550, 21.2693, 21.6216, 21.3376, 21.6500],
    'Longitude': [-157.6976, -157.7448, -157.7151, -157.7392, -
157.8391, -157.8311, -157.6727, -157.6938, -157.8467, -157.7390, -
157.8248, -157.6601, -158.1117, -158.1883,  -157.8255, -157.8069, -
157.8232, -158.0820, -158.1251, -158.0628],
    'Positive_Samples': [2, 3, 4, 1, 2, 4, 2, 1, 10, 3, 4, 1, 2, 1, 1,
2, 3, 1, 1, 1]
}
df = pd.DataFrame(data)

#Convert to GeoDataFrame
gdf = gpd.GeoDataFrame(df, geometry=gpd.points_from_xy(df['Longitude'],
df['Latitude']), crs="EPSG:4326")

#Convert to Web Mercator projection
gdf = gdf.to_crs(epsg=3857)

#Sort Data by Positive Samples (Smallest to Largest)
gdf = gdf.sort_values(by="Positive_Samples", ascending=False)  #
**Larger dots first, smaller dots last**

#Coloring**
unique_beaches = gdf['Beach'].unique()
colormap = plt.colormaps["gist_ncar"]  # Large colormap with unique
colors
color_values = np.linspace(0, 1, len(unique_beaches))  # Evenly space
colors
```

```
beach_colors = {beach: colormap(color_values[i]) for i, beach in
enumerate(unique_beaches)}

#Scale for Dot Sizes
dot_scale = 65  # Scaling factor for dots

#Main Figure
fig, ax = plt.subplots(figsize=(12, 10))

#Plot each beach with its assigned color
for _, row in gdf.iterrows():
    ax.scatter(
        row.geometry.x, row.geometry.y,
        s=row['Positive_Samples'] * dot_scale,  # Use the same scale
for dots
        color=[beach_colors[row['Beach']]],
        edgecolor='black', linewidth=1.5,  # Black outline
        alpha=0.9
    )

#For visibility
ctx.add_basemap(ax, source=ctx.providers.CartoDB.Positron,
zoom_adjust=-1)

#Legend with Frequency Next to Beach Name
handles = [
    plt.Line2D(
        [0], [0],
        marker='o',
        color=beach_colors[beach],
        markersize=np.sqrt(gdf[gdf['Beach'] ==
beach]['Positive_Samples'].values[0]) * 10,
        markeredgecolor='black',
        markeredgewidth=1.5,
        linestyle='',
        label=f"{beach} ({int(gdf[gdf['Beach'] ==
beach]['Positive_Samples'].values[0])})"
    )
    for beach in unique_beaches
]

ax.legend(handles=handles, loc='upper left', bbox_to_anchor=(1, 1),
title="Beach Names (Positive Sample Count)", fontsize=20,
title_fontsize=25, prop={'size': 20,'weight': 'bold'}, frameon=False)

#Remove unnecessary ticks & frame
ax.set_xticks([])
ax.set_yticks([])
ax.set_frame_on(False)

#Adjust title
ax.set_title("Enterococci-Positive Water Samples on Oʻahu Beaches",
fontsize=25, fontweight='bold')

fig.savefig("/Users/sierralopez/Documents/oahu_map.png", dpi=300,
bbox_inches='tight')
plt.show()
```

Code S2: R Code used to calculate Pearson correlation coefficients and generate scatterplots assessing the relationship between enterococci detection rate and the detection rate of CrAssphage and HF183 across beach sampling sites (Figure 9).

```
library(tidyverse)
library(ggpubr)
library(patchwork)
library(gt)

beach_data <- tibble(
  Beach = c("Waimanalo", "Kawaikui", "Lanikai", "Kalama", "Kahanamoku",
"Moana", "Sandy", "Hanauma", "Ala Moana", "Kailua",
            "Kuhio", "Makapuu", "Kapaeloa", "Pokai Bay", "Tavern
Beach", "Diamond Head Showers", "Kapiolani", "Chuns Reef",
            "Kohola Lagoon I", "Shark's Cove"),
  Enterococci = c(2, 3, 4, 1, 2, 4, 2, 1, 10, 3, 4, 1, 2, 1, 1, 2, 3,
1, 1, 1),
  CrAssphage = c(0, 0, 2, 0, 1, 4, 1, 0, 7, 2, 2, 0, 0, 1, 1, 0, 0, 0,
0, 0),
  HF183 = c(0, 0, 1, 0, 1, 2, 1, 1, 5, 1, 2, 1, 0, 0, 0, 0, 2, 0, 1, 0)
)

plot_crass <- ggplot(beach_data, aes(x = Enterococci, y = CrAssphage))
+
  geom_jitter(width = 0.1, height = 0.1, color = "blue", alpha = 0.6,
size = 3) +
  geom_smooth(method = "lm", se = FALSE, color = "blue") +
  stat_cor(method = "pearson", label.x = 1, label.y = 6.5) +
  labs(title = "CrAssphage vs Enterococci",
       y = "CrAssphage Detections Per Beach") +
  theme_bw() +
  theme(axis.title.x = element_blank())

plot_hf <- ggplot(beach_data, aes(x = Enterococci, y = HF183)) +
  geom_jitter(width = 0.1, height = 0.1, color = "red", alpha = 0.6,
size = 3) +
  geom_smooth(method = "lm", se = FALSE, color = "red") +
  stat_cor(method = "pearson", label.x = 1, label.y = 4.5) +
  labs(title = "HF183 vs Enterococci",
       y = "HF183 Detections Per Beach") +
  theme_bw() +
  theme(axis.title.x = element_blank())

combined_plot <- plot_crass + plot_hf +
  plot_annotation(
    caption = "Enterococci-Positive Samples Per Beach (x-axis shared)",
    theme = theme(plot.caption = element_text(hjust = 0.5, size = 10))
  )

combined_plot
```

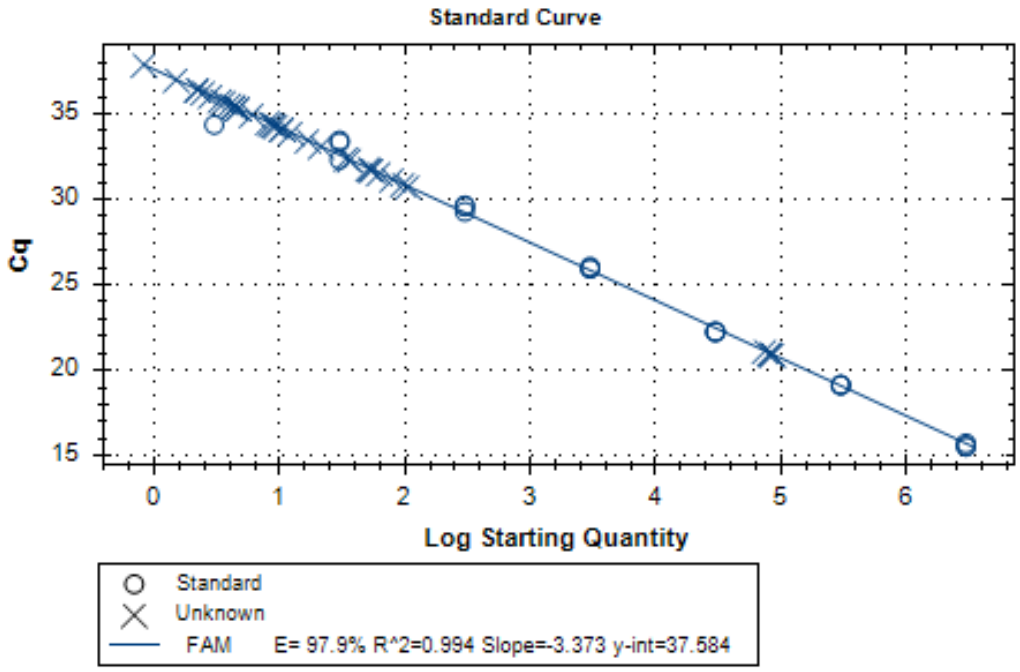

Figure S1: Standard curve for CrAssphage quantification by qPCR, displaying the relationship between Cq (cycle threshold) and the log starting quantity of DNA. The provided slope, y-intercept and efficiency (E=97.9%) indicate the performance of the qPCR assay. Samples 1-25.

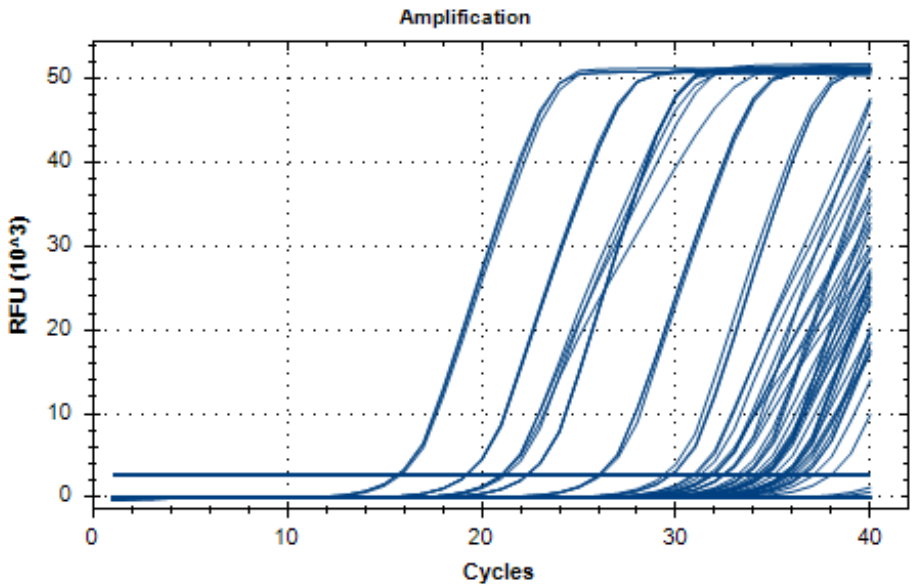

Figure S2: Amplification curves for CrAssphage qPCR assay, showing fluorescence intensity (RFU) over 40 cycles. Each curve is representative of a sample (1-25)

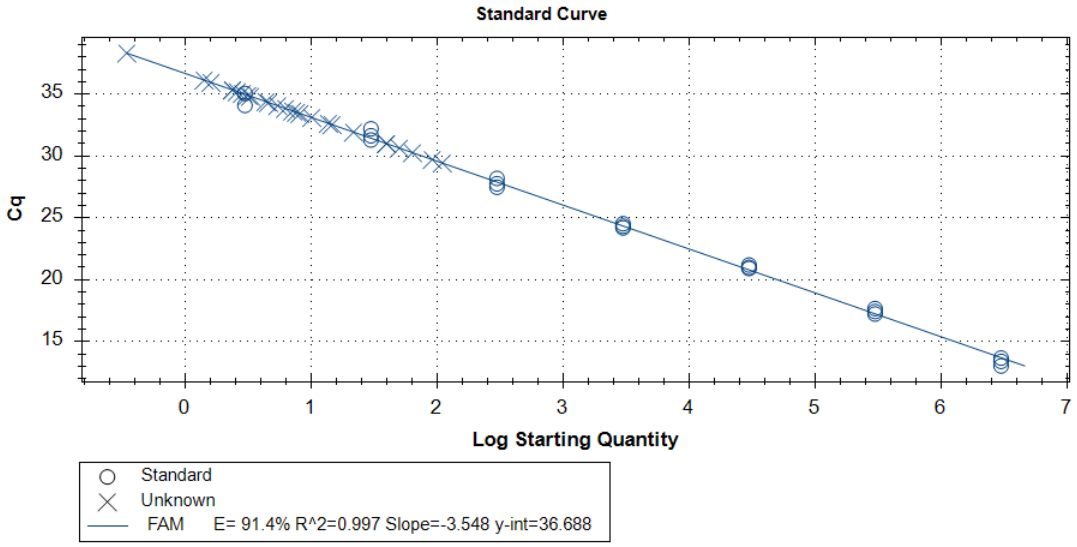

Figure S3: Standard curve for CrAssphage quantification by qPCR, displaying the relationship between Cq (cycle threshold) and the log starting quantity of DNA. The provided slope, y-intercept and efficiency (E=91.4%) indicate the performance of the

qPCR assay. Samples 26-49.

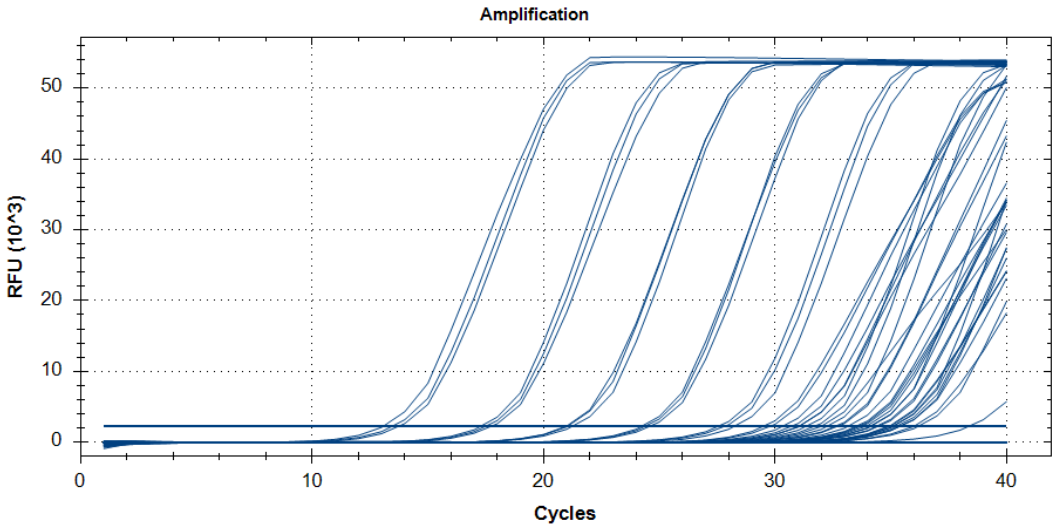

Figure S4: Amplification curves for CrAssphage qPCR assay, showing fluorescence intensity (RFU) over 40 cycles. Each curve is representative of a sample (26-49).

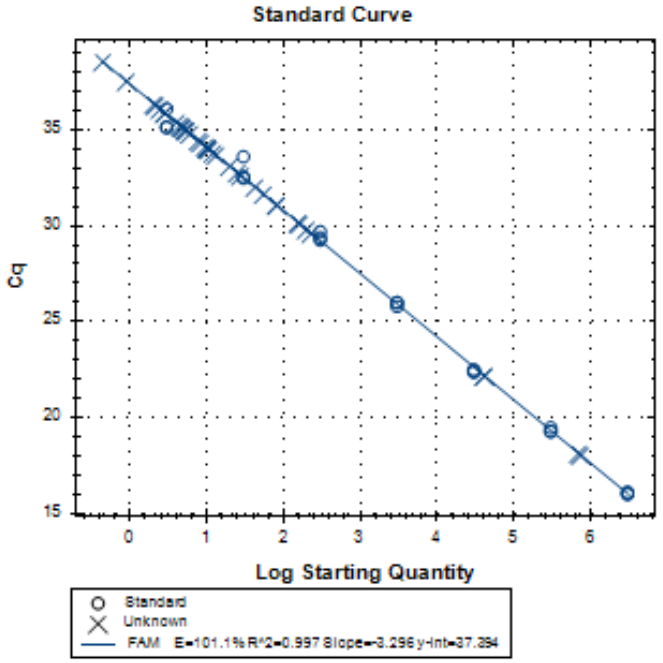

Figure S5: Standard curve for HF183 quantification by qPCR, displaying the relationship between Cq (cycle threshold) and the log starting quantity of DNA. The provided slope, y-intercept and efficiency (E=101.1%) indicate the performance of the qPCR assay.

Samples 1-26

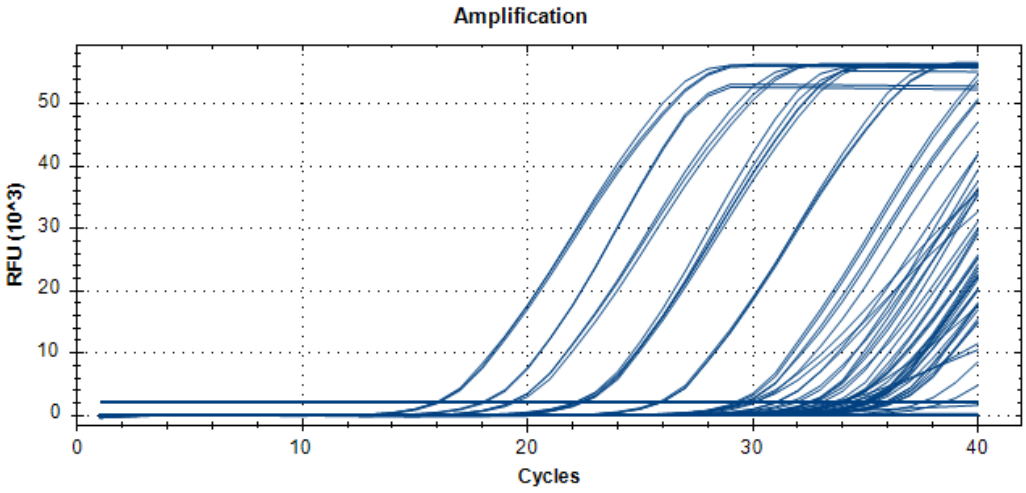

Figure S6: Amplification curves for HF183 qPCR assay, showing fluorescence intensity (RFU) over 40 cycles. Each curve is representative of a sample (1-26).

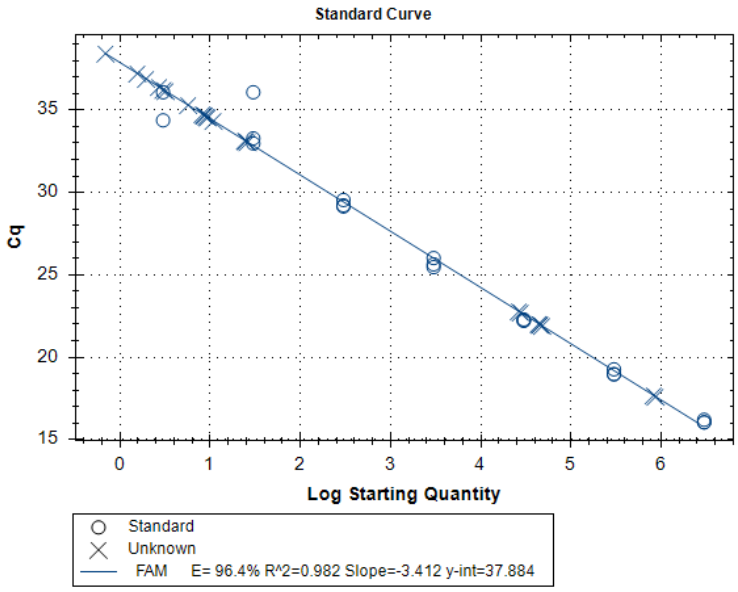

Figure S7: Standard curve for HF183 quantification by qPCR, displaying the relationship between Cq (cycle threshold) and the log starting quantity of DNA. The provided slope, y-intercept and efficiency (E=96.4%) indicate the performance of the qPCR assay.

Samples 27-49.

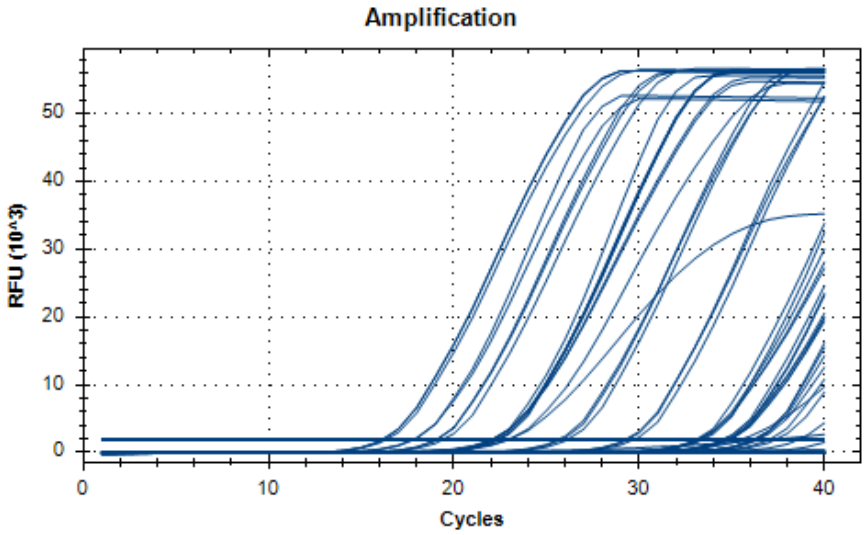

Figure S8: Amplification curves for HF183 qPCR assay, showing fluorescence intensity (RFU) over 40 cycles. Each curve is representative of a sample (27-49).

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