CHALLENGES WITH THE APPLICATION OF QPCR BASED TESTS FOR ENTEROCOCCI AS A RAPID BEACH MANAGEMENT TOOL IN HAWAI'I

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I certify that we have read this thesis and that, in our 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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To my parents, Georgio, Janel, Ahmet, Sabine, Bert and my friends and family.

Thank you for all your support and inspiration throughout this journey, I could not have done this without you. Mahalo for believing in me and being patient.

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ABSTRACT

Rapid quantitative PCR based methods (USEPA Method 1609 and 1611) that can evaluate water quality within a few hours are important since urbanization and our changing climate will alter inputs of bacteria into the environment, which can compromise the health of Hawai'i residents and visitors. We identified that assay interference in Hawaiian coastal waters is associated with the DNA extraction process, but not with the PCR amplification procedure. Further we demonstrated that acidification of water samples can alleviate assay interference associated with Hawai'i's beach water samples.

It is speculated that acidification of coastal water samples helped to dissolve calcium carbonate rich coralline particles, common to subtropical and tropical coastal waters, hence eliminating a substratum for DNA to bind. This study envisions, that once the assay can be used to reliably quantify microorganisms (such as enterococci, human-associated *Bacteroides*), these tests will be used for beach water quality evaluations at popular beaches in the state of Hawai'i.

In this research, water matrixes for PCR inhibitors were tested from water samples gathered at selected beaches on Oahu, by using the EPA Methods 1609 and 1611, Enterococci in Water by TaqMan ®Quantitative Polymerase Chain Reaction (qPCR) with Internal Amplification Control (IAC) Assay. There is a relationship between the sodium chloride and calcium carbonate on Oahu, and the inhibition of the rapid method, used to identify enterococci. The corals in Hawai'i are unique and add in with the water's composition that is interfering with the DNA extraction process, and underestimating the rapid method results.

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1.0 INTRODUCTION

1.1 Background

Climate change is expected to alter precipitation patterns and storms in many areas, producing more often intense rainfalls (Strauch et al., 2014). This will result in shifts in the quantity and timing of surface water runoff delivered to streams and nearshore regions, which is projected to affect watershed processes and the health of coastal environments (Strauch et al., 2014). In addition, pollutant loads are expected to increase due to population growth (WRRC, 2006).

Recent modeling efforts have indicated that these shifts in rainfall patterns combined with an increased urbanization will change the amount of fecal indicator bacteria (FIB) in tropical waters (Strauch et al., 2014; Fujioka, 2015). Compromised sewage overflow systems and increased runoff can make it unsafe for residents and visitors to use the beaches for recreational activities and even scare off people (Hawai'i, 2015). Concentrations of enterococci in coastal waters may be higher after rainfall even in the absence of sewage contamination (Tomlinson, 2011).

Tourism is the main income for the major Hawai'ian islands, especially Oahu with Honolulu as its' capital and Waikiki being one of the most famous beaches (Hawaii, 2015). Therefore, better beach water quality monitoring programs are urgently needed (Pencheon, 2012; Fujioka, 2015).

The Clean Water Act and Beaches Environmental Assessment and Coastal Health (BEACH) Act requires states along a coast to monitor recreational waters for fecal indicator bacteria (FIB) and to evaluate the water quality (Tomlinson, 2011). Earlier studies, including the US Environmental Protection Agency, pointed out that people are at higher risk of becoming ill from pathogens in fecal matter when swimming in water contaminated with fecal pollution (Tomlinson, 2011; Soller, 2010; Harwood, 2014), while current indicator bacteria can originate from any source (Fujioka, 2015). Therefore, due to the shift in climate patterns, coupled with population growth, there is a need for better water monitoring programs which would utilize fast and precise tests to link water quality parameters to human health risk.

1.1.1 Oahu sewage spills

An example for excessive rainfall, is the last week of March in 2006, when Honolulu's Manoa Valley experienced 42 consecutive days of rainfall (WRRC, 2006). Because of the rain-intensity, infiltration of rainwater reached its limits and water from the saturated ground ran into the sanitary sewer system, which contributed to a rupture of a 42-inch pipe, in the heart of Waikiki (WRRC, 2006). The City had to divert the sewage into the Ala Wai canal, which forms the northern and western boundaries of Waikiki (Tomlinson, 2011), to avoid sewage flowing into Waikiki. It took six days to fix the damage, and within this time, 48 million gallons of sewage ended up in the Ala Wai canal, becoming one of the largest sewage spills in the history of Oahu (WRRC, 2006).

The State Health Department and City & County of Honolulu found increased amounts of enterococci in the Ala Wai canal, as well as around Waikiki beach (WRRC, 2006). A tragedy happened when a resident fell into the canal and became infected with *Vibrio vulnificus*, a flesh-eating bacterium native to marine waters in Hawai'i, who then passed away within a few days (WRRC, 2006). This event raised questions about the risks of going to beaches that have been exposed to sewage spills, for example what factors could help determine how quickly the bacteria levels returned to safe ones (WRRC, 2006).

In the last couple of years, accidents have been happening frequently. One example is a spill that occurred in 2015, again around Waikiki, when 129,000 gallons of wastewater flowed into the ocean (Hawaii, 2015), because the city's sewage system could not handle the storm water. In addition, a sewage pumping station for that area was under construction, leaving the sewage system without sufficient capacity to handle the excess waters (Hawaii, 2015). Leaves and other debris were also blocking storm drains around the same time. Because it is a reoccurring problem, daily monitoring is necessary to be able to inform of safe water quality, for recreational use.

1.1.2 Enterococcus as an indicator

Federal recreational water quality criteria and related water quality standards issued by individual states are based on epidemiological studies, which have identified a relationship between the concentrations of enterococci and the risk of gastrointestinal illness, related to recreational water use (Wade et al., 2010; Fujioka, 2015).

Enterococci is a standard bacterial indicator for determining the extent of fecal contamination of recreational surface waters and the possible presence of enteric pathogens (Colford et al., 2012).

Sample analyses can take up to 24-48 hours and polluted beaches could remain open while the analyses are completed, which means that the contamination event may have passed by the time warnings can be posted (Colford et al., 2012).

1.2 Advantages through qPCR

Molecular methods, such as the quantitative Polymerase Chain Reaction (qPCR), can identify fecal indicator organisms faster than cultivation based methods, such as membrane filtration or substrate kits, by measuring and targeting specific genetic markers (Wade et al., 2010; Colford et al., 2012).

Method 1609 and Method 1611 describe a qPCR procedure for monitoring water quality, based on the amplification and detection of a specific region of the large subunit ribosomal RNA gene, from enterococci (Nobel et al., 2010). While other methods require 1 to 2 days to obtain results, outcomes by this method can be obtained within 2 to 4 hours (Wade et al., 2010). This advantage allows notifications of water quality to be available on the same day, preferably at a time before most people arrive to a beach (Wade et al., 2010). Also, beach notifications can be removed in timely fashion once tests have indicated a satisfactory water quality.

1.3 QPCR issues in Oahu, Hawai'i

The use of molecular methods has been unsuccessful in Hawaiian waters (Kirs, 2015). An earlier study which analyzed 121 samples, collected over a one year period, on twelve beaches of Oahu, has identified that there is an issue with the application of rapid qPCR based methods in Hawai'i. Roughly 70% of the samples mentioned could not be analyzed for enterococci using this technique (Kirs, 2015).

The problem appeared to be related to interference resulting from samples clogging the filters during the concentration step, or due to the loss of DNA during the extraction step. In addition, DNA amplification, may be interfered by compounds that inhibit the enzymatic activity of the Taq DNA polymerase and/or by interfering with the annealing of the primer and probe oligonucleotides to sample target DNA, enzyme and/or by the quenching of hydrolyzed probe fluorescence (USEPA 2013 Method 1609).

Since further dilution of the samples did not alleviate the tests interference, and as freshwater samples did exhibit interference, loss of DNA during the extraction step seems to be the main issue (Kirs, 2015).

1.4 Objectives of Study

The overarching goal of this study was to provide a modified sample analyses protocol so rapid qPCR based tests could be utilized for better protection of residents and visitors in Hawai'i.

To achieve this goal following objectives were set:

1) identifying the cause of interference,

2) based on the outcome of objective 1, modifying the analyses protocol as needed, by testing alternative sample treatment options (in this case pH-adjustment).

The cause of interference was tested to identify whether the problem appeared during any DNA extraction steps or if it was related to particles in the seawater, interfering with the qPCR machine process, during amplification.

2.0 METHODS

Several experiments were executed before narrowing the tests down to the two objectives below (Appendix A, Appendix B). Sections of the EPA methods 1609 and 1611, relevant to the qPCR issues studied, were used for the following experiments and are detailed below.

2.1 Objective 1: Interference Experiment

For objective 1 several tests were done until the focus was narrowed down to comparing the control DNA in alternative steps of extraction. Followed up, the control DNA was added to the water samples, by mixing it in with the AE Buffer needed for extraction, and compared to the control DNA added into the mastermix (Section 2.3). The mastermix is a premixed solution containing TaqMan® Environmental Master Mix 2.0, Forward and Reversed Primer, Probe, Bovine serum albumin (BSA) at optimal concentrations for efficient amplification of DNA templates by qPCR. To identify whether DNA is lost during the extraction or if there are qPCR inhibitors in the Hawai'ian seawater, which interfere with the qPCR assay, the following set of experiments were conducted.

On September 26th, October 14th, and October 20th of 2016, one liter of seawater from each sample site (Figure 1, Table 1) were collected in the morning of each trial, along the coastline, in 30-40 cm deep water.

In the laboratory, two 100 ml sample portions from each beach sample were filtered as described below (Section 2.2). For each water sample, one filter was prepared by seeding control DNA into the extraction buffer (AE-Buffer), while a second filter was prepared using extraction buffer without seeded control. For the second filters the control DNA was added later to the mastermix (Table 3) at final concentrations (0.01 ng/ml) used for qPCR (Section 2.5).

2.1.1 Map of Sample Sites on Oahu, Hawai'i

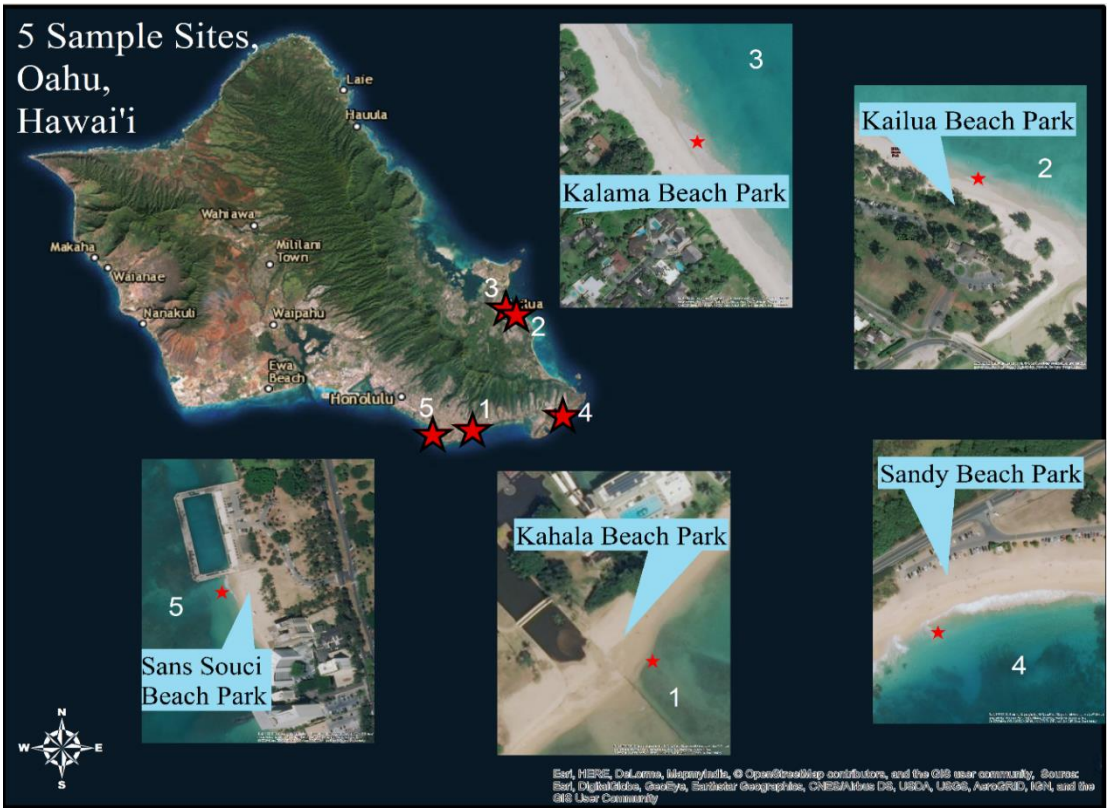

Figure 1: Map of the Island Oahu, Hawai'i, including Sample Sites.

Seawater was collected in the mornings from 5 beaches (Table 1) along the North-East, South-East and the South of Oahu, in 30-40 cm deep water. The location of the 5 sample sites for objective 1 and 2 are marked with red stars in the map above.

2.1.2 Sample Site Locations

Table 1: Five Sample Sites for Objective 1 and 2 on Oahu, Hawai'i

BEACH SITE	LOCATION
Sandy Beach Park, Hawai'i Kai, Oahu, Hawai'i	Latitude: N21° 2857", Longitude: W157° 6727"
Kailua Beach Park, Kailua, Oahu, Hawai'i	Latitude: N21° 3976", Longitude: W157° 7271"
Kalama Beach, Kailua, Oahu, Hawai'i	Latitude: N21° 4046", Longitude: W157° 7397"
Sans Souci Beach, Waikiki, Oahu, Hawai'i	Latitude: N21° 2683", Longitude: W157° 8225"
Kahala Beach, Waialae, Oahu, Hawai'i	Latitude: N21° 2701", Longitude: W157° 7762"

2.2 Filtering the Beach-Water Samples

For both objectives, water samples were collected at the 5 sample sites (Table 1) with sterile bottles and transported in a cooler designated for the lab. A 4°C refrigerator was used to store the water samples.

To capture the DNA onto a membrane filter, sterilized funnels were attached to a vacuum manifold. All funnels were prepared with a white, sterile, 0.45 µm pore size polycarbonate membrane filter, 47mm in diameter, by using two pairs of forceps, straight or curved, with smooth tips, to handle the filters without damaging them. For a negative extraction control 100 ml of sterile Milli-Q® Integral Water (Millipore, Billerica, MA) was used instead of sea water.

Next 100 ml of each beach sample were measured in graduated, autoclaved or UV-sterilized cylinders, before added to the funnels. The vacuum was turned on to start filtration. When the filter was visibly dry, the sides of the funnel were rinsed with a small volume of the phosphate buffered saline (PBS) solution. When the filter was dry again, the vacuum was turned off and the funnel was removed from the base carefully without disturbing the membrane. The two pairs of forceps, mentioned before, were sterilized by soaking them in ethanol and then applied to a flame. When the forceps were cooled, the filter was carefully folded in half, making sure to handle the filter by its edges.

The filter was then folded in half twice again and placed in the, semi-conical screw cap microcentrifuge 2.0 ml extraction tubes, prepared in advance with 0.30 mg ± 0.01 mg of acid washed glass beads, of 212−300 μm diameter. All tubes were labeled with a permanent ink marker to avoid confusion of the sample sites.

2.3 Preparation of Mastermix

The mastermix composition (Table 3), for the control DNA-assay and the enterococcus-qPCR-assay were prepared the day of the experiments. For objective 1 the control DNA (Salmon Sketa, 0.1 μg/ml) was added either to the filters with the AE Buffer before the bead beating, or the mastermix itself (final concentration 0.25 ng/μl), which was added to the wells with the sample in the 96 well tray (Section 2.5).

2.3.1 Primer and Probe Sequence

Table 2: Primer and Probe Sequences for Sketa and Enterococci Assay

Assay	Forward Primer	Reverse Primer	TaqMan® Probe	Reference
Salmon Sketa	5'-GGT TTC CGC AGC TGG G-3’	5'-CCG AGC CGT CCT GGT C-3’	[6-FAM]-5'-AGT CGC AGG CGG CCA CCG T-TAMRA	(USEPA 2012 Method 1611)
Enterococcus	5'-GAG AAA TTC CAA ACG AAC TTG	5'-CAG TGC TCT ACC TCC ATC ATT	[6-FAM]-5'-TGG TTC TCT CCG AAA TAG CTT TAG GGC TA-TAMRA	(USEPA 2012 Method 1611)

[6-FAM]- 6-FAM™ Fluorescein, Salmon DNA primer and probe sets were saved in a freezer, -20°C or -80°C.

2.3.2 Sample Dates and Methods

Table 3: Sample Dates and Methods for Objective 1 and 2

DATES	EXPERIMENT	AE-BUFFER	qPCR REACTION
09/26/16 10/14/16 10/20/16	**Objective 1:** Control DNA added to PCR mastermix	600 µl AE-Buffer	TaqMan® Environmental Master Mix 2.0, Forward and Reversed Primer (1000 nM each), Bovine serum albumin (BSA) (0.2 mg/mL), control DNA (0.4 ng/µl), Probe (80 nM),
	Control DNA in Extraction Buffer	590 µl AE-Buffer and 10 µl control DNA	Mastermix without control DNA
01/25/17 02/13/17 03/20/17	**Objective 2:** Adjusted pH level from 5 pH to 2.5 pH	590 µl AE-Buffer and 10 µl control DNA	Mastermix without control DNA

The AE-Buffer (10 mM Tris-Cl, 0.5 mM EDTA; pH 9.0) or buffer mix was added to the sample filter before the bead beating process (section X.X). The control DNA used was Salmon Sketa (0.01 ng/ml). The mastermix here is the PCR Assay Mix, composed as seen above with a final concentration of 0.25 ng/µl, used for the Optical 96 well PCR reaction trays.

2.4 Extraction of DNA

The AE-Buffer used for the bead beating process was prepared either with or without control DNA (Table 3), depending on the purpose of the test. Before each use, the buffer mix was vortexed and centrifuged down, in a micro-centrifuge tube. 600 µl of the buffer or the buffer mix (590 µl AE-Buffer and 10 µl control DNA) was added to each new filter tube. The pipette tip was replaced each time to prevent a crossover contamination.

Next the tubes were inserted into a multi-place bead beater (Mini-BeadBeater-8, Biospec Products Inc. 3110BX or equivalent). The tubes were distributed evenly to prevent damage to the instrument. Then the safety hood was closed and the bead beater ran at maximum speed for 2 minutes. When the instrument stopped, the tubes were removed, and centrifuged for 1 minute at 12,000 x g in a microcentrifuge.

Meanwhile, a fresh sterile 1.7 ml microtube was prepared and labeled for each sample. Using a micropipette, and changing tips between each sample, 300 µl from each of the centrifuge-tubes was transferred to the corresponding fresh labeled tube. The filter and beads were not jostled to prevent picking up unwanted debris particles. Then the tubes containing the recovered liquid were centrifuged once more for 5 minutes, at 12,000 x g.

Again, a new sterile 1.7 ml microtube for each sample was prepared. This time 200 µl from the freshly spun batch were transferred carefully into the new prepared tubes, without touching the precipitate.

This procedure was repeated a last time, transferring 50 µl into a last set of prepared tubes. These sets of final samples were diluted with 200 µl of just AE-Buffer and vortexed to mix. This sample extract, noted 25X, was used for the qPCR analyses in effort to determine the concentrations of existing DNA.

2.5 Quantitative Polymerase Chain Reaction

2.5.1 Loading the qPCR Plate

The assays were run using a white Optical 96 well PCR reaction tray (ABI N801-0560). The wells were labeled accordingly to each sample. Using a repeating pipette, 20 µl of the appropriate master mix was added to the wells designated for that assay. Next 5 µl of the samples marked 25 X (Section 2.4) were added, from the last set of test tubes, into the wells containing the mastermix. Each tube had been vortexed and spun, before removing the DNA extract and adding it into the wells of the qPCR plate, while changing pipette tips between each well. Each sample was run in duplicate for its assays.

2.5.2 Sealing the qPCR Plate

To seal the qPCR plate, an adhesive seal was placed on the top of the plate, and pressure was applied, to ensure that each well was completely sealed. The plate was placed in a balanced plate spinner and spun for 20 seconds.

2.5.3 Initiating the qPCR Run

All the reactions were incubated in the CFX96^{TM} Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA). The lid was opened and closed by using the controls on the instrument, to avoid damaging the machine. The CFX manager software on the attached computer was opened and “create new experiment” selected.

The cycling parameters were set to 95 degrees for 10 minutes to activate the enzyme, followed by 40 cycles of 95 degrees for 15 seconds, and 60 degrees for 2 minutes. The fluorescence generated by TaqMan probes in each reaction was measured at the end of the DNA extension step (60°C incubation) in each cycle and quantification was performed using software (Bio-Rad CFX Manager 3.1) defaults.

The plate-setup was defined by identifying sample type and assay in each well, on the plate-editor screen. After pressing start, the assay was named and dated accordingly. When the run was finished the Optical 96 well PCR reaction tray was removed from the thermocycler and discarded without breaking the seal. The data was exported from the thermocycler to the pre-prepared spreadsheet, to calculate the results.

2.6 Calculations

The assay interference was measured by comparing fluorescence threshold values (C_t) determined by the CFX96^{TM} Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA) in pure water and beach water samples. The average of every duplicate for each beach was calculated and subtracted from the average of the pure control water.

This difference of the threshold cycle ($C_{t\ pure} - C_{t\ beach}$) is referred to as ΔC_t and used to indicate assay interference. Samples not compromised by tests interference have a ΔC_t equal to zero, while heavily compromised samples (more than 10 times underestimation of seeded control DNA concentration) have a $\Delta C_t \geq 3.3$. These ΔC_t values were compared to each other, from the three runs within each objective.

2.7 Objective 2: Modifying the Analyses Protocol by Altering pH-levels

For objective 2, the pH concentrations of each beach sample were altered to lower levels, 5 through 2.5 pH, to identify whether acidification can eliminate or neutralize calcium carbonate rich coralline materials present in the sample, which might be inhibiting the procedure.

In these three sets of experiments, beach water samples were seeded with control DNA as well as with overnight culture of *Enterococcus faecalis* (ATCC® # 29212™) stock culture. *E. faecalis* was seeded to explore whether control DNA and Enterococcal DNA behave in a similar manner when experimental conditions are modified. 100 ml subsamples were acidified in the laboratory to pH 5.0, pH 4.0, pH 3.0 and pH 2.5 using 6N hydrochloric acid.

The samples for these tests were gathered on January 25th, February 13th, and March 20 of 2017 (Figure 1, Table 1). In addition, one of the two non-acidified samples were rinsed with 100 ml Milli-Q® Integral Water (Millipore, Billerica, MA) in the last two runs, to identify whether high salt concentrations interfered with the extraction. Acidified and non-acidified subsamples were filtered (Section 2.2), extracted (Section 2.4) and tested by qPCR (Section 2.5). The pure water served again as an interference free control.

To fulfill objective 2, 10 µl of the control DNA (0.01 µg/ml) was always added to the filter through the AE Buffer before being beat processed (Table 3), and the pH of the 100 ml samples were altered (Appendix G) as stated above. Enterococci was measured by the cells pre-quantified standards using assay 1609. The protocol for enterococci and control DNA were close to identical.

3.0 RESULTS

3.1 Experiments for Objective 1

The first set of experiments were conducted to identify whether the assay interference was associated with the DNA extraction or with the PCR amplification.

Table 4: Average of ΔC_t for all 3 Experiments of Objective 1

Beach (B.)	Δ C_t First Run		Δ C_t Second Run		Δ C_t Third Run	
	Control in Sample	Control in PCR Mastermix	Control in Sample	Control in PCR Mastermix	Control in Sample	Control in PCR Mastermix
Kahala B.	1.5	-1.6	-5.7	0.4	-0.7	-1.2
Kailua B.	-8.0	-0.8	-1.1	-0.1	-5.0	-1.9
Kalama B.	-7.8	-1.8	-7.2	-0.3	-1.2	-1.8
Sandy B.	0.3	-1.6	-0.3	-0.1	-0.1	-1.1
Sans Souci B.	1.3	-1.4	-9.7	0.0	1.4	-1.9

Control DNA was either added to the beach-water sample or to the PCR mastermix. The ΔC_t of all three trials were calculated from each run and each beach. Red indicates compromised samples.

Six out of fifteen samples (40%) for "control in sample" (Table 4) tested exhibited DNA loss during the DNA extraction step ($\Delta C_t \geq 3.3$), which would have led to a more than 10-fold underestimation of DNA concentrations, while none of these samples appeared to contain compounds inhibiting PCR reactions to this extent.

The results indicate that the assay interference is associated with the DNA extraction step, because when the control DNA was seeded into the PCR mastermix after the extraction step, the interference was limited (Table 3).

This suggests that DNA is lost during the DNA extraction step, likely due to precipitation with the suspended particles or degraded by the enzymes such as deoxyribonucleases. Based on the limited number of samples tested, the interference did not appear to be consistently associated with any one beach, but more samples are need.

3.2 Experiments for Objective 2

The second set of the experiments was conducted to identify whether acidification can help eliminate the interference by removing calcium carbonate rich coralline particles, so that the DNA will not have any substrate to bind to, and possibly precipitate within. This was done to avoid DNA falling to the bottom of the tubes before extracting final liquid for use in the qPCR tray (Section 2.5.1).

The research project suggests that acidification alleviates assay interference associated with the Hawaiian coastal waters (Table 5). There was a strong significant linear correlation (n=14, R^2=0.94, P<0.001) (Figure 2) between the assay interference estimates for seeded enterococci and control DNA, indicating that both are affected by the tests interference in a similar manner.

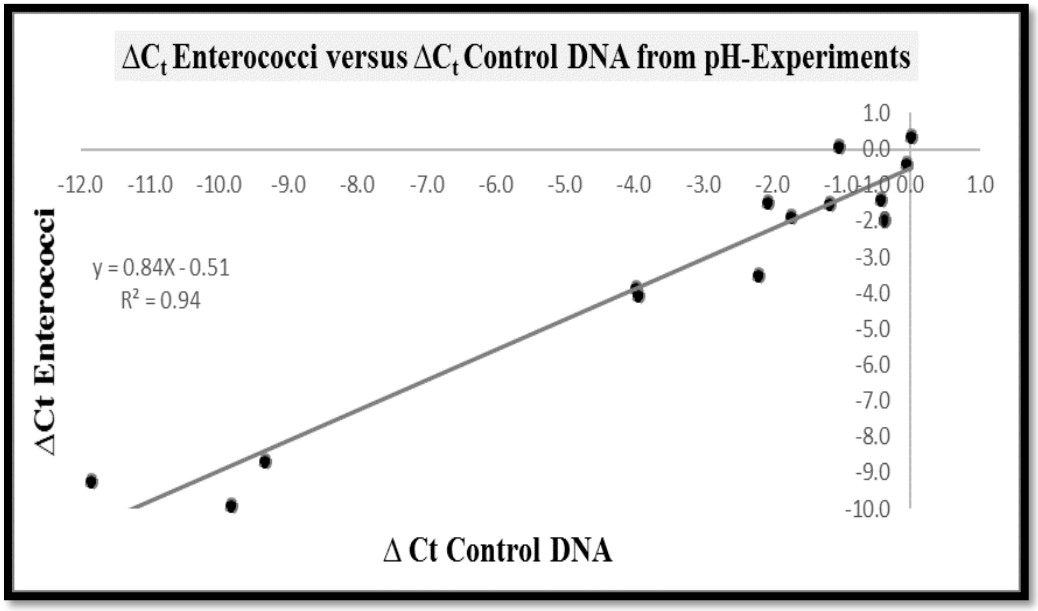

Figure 2: Graph of ΔC_t Enterococci versus ΔC_t Control DNA of the pH Adjustment Experiments, (R^2=0.94, P<0.001)

Spearman rank correlation analyses indicated that the pH adjustment had a significant effect on the interference (n=147, R=-0.396, P<0.001) when the data for enterococcal DNA and control DNA were pooled from all five beaches and considering all three trials for this part of the experiment (Figure 3).

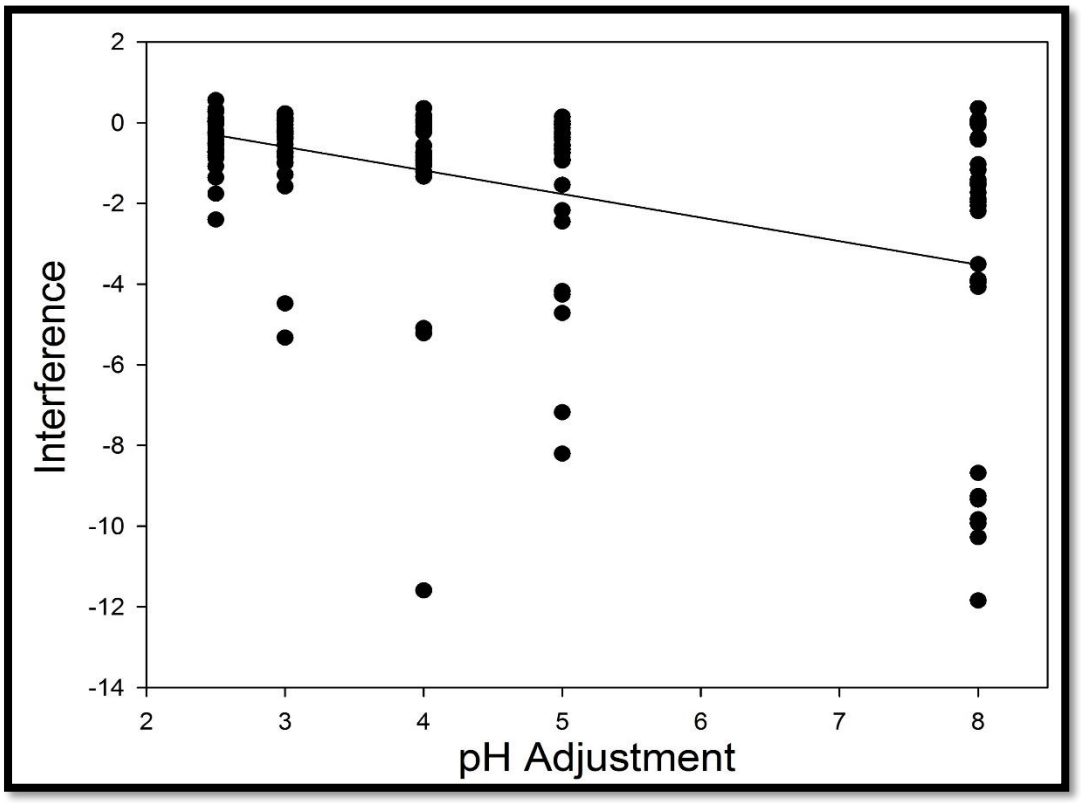

Figure 3: Graph of Interference versus pH Adjustment

As it is shown in the graph above, the more the pH is adjusted towards 4, 3, and 2 pH, the less interference appears. The values on the y axis are moving closer to zero with lower pH levels, which is the goal of limiting the interference

The pH Adjustment had significant effect on the interference when data for control DNA and enterococci were analyzed separately as well (n=72, R=-0.447, P<0.001 and n=75, R=-0.364, P=0.001 respectively) (Figure 4).

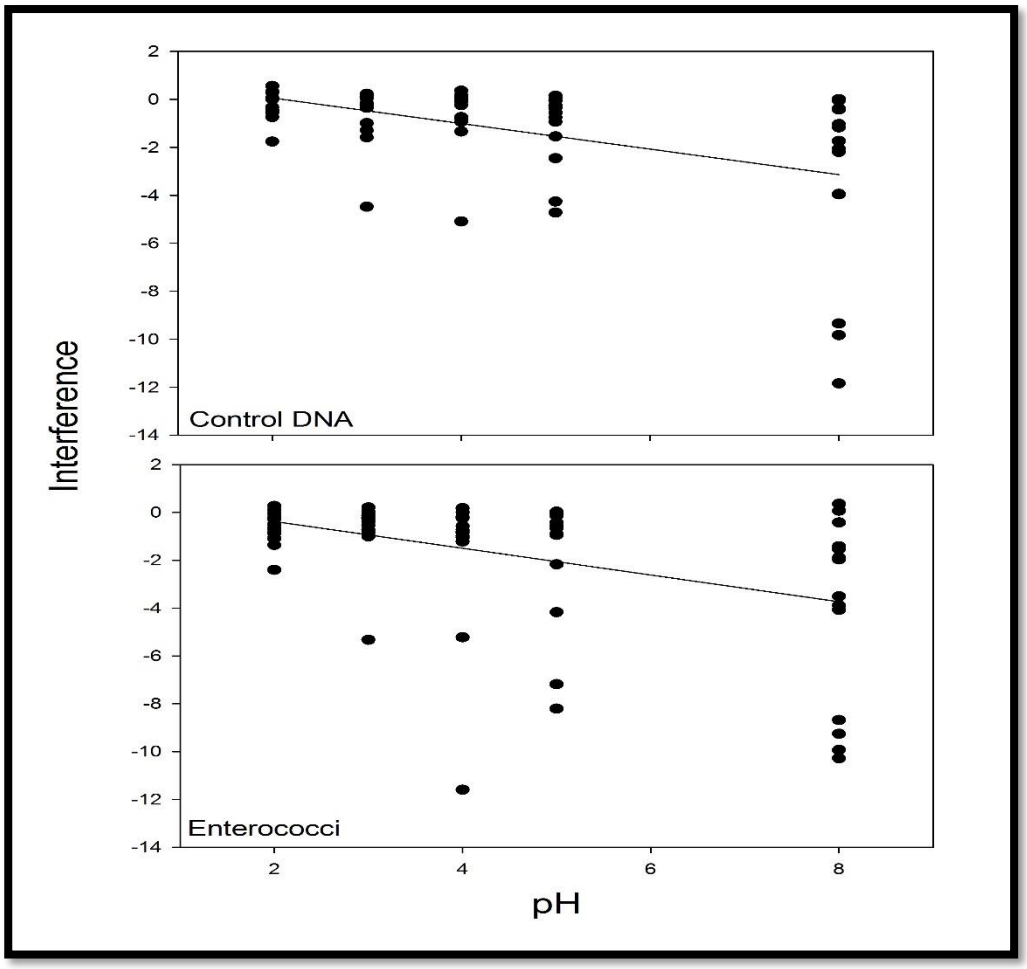

Figure 4: Effect of pH Adjustment on the Interference for Control DNA and Enterococcus DNA.

In experiments two and three (Table 5) the samples that were rinsed additionally with pure water did not show a significant difference overall in results, except one out of five that were inhibited. The red values indicate interference (Table 5) at sample site, which showed the most at Kalama Beach, and was resolved in lowering the pH value to 4 in the first trial, 3 pH in the second, and 2.5 pH for the third trial.

Interference was shown in 46.7 % of samples (n=15) with enterococci and 40.0 % of samples with control DNA by having a $\Delta C_t \geq 3.3$ (10-fold difference). 80.0 % of samples seeded with enterococci and 73.3 % with control DNA had a $\Delta C_t \geq 1$ (2-fold difference). By changing the acidity, the interference decreased. The difference of $\Delta C_t \geq 3.3$ post adjustment was reduced to 6.7 % in both cases (sample with enterococci or control DNA), as well as reduced to 20.0% and 6.7 %, for the $\Delta C_t \geq 1$ values.

Most improvement was achieved in the case of $\Delta C_t \geq 1$ for original pH, with enterococci added to the samples, and an adjusted pH level to 3, with a difference of 73.3 %. Overall each trial showed improvement. It is more affective to compare the results with 3 pH because it is difficult to adjust the pH as low as 2.5.

High salinity and suspended coralline particles, associated with the Hawaiian marine water samples, are implicated in the loss of DNA during the DNA extraction step. It is speculated that DNA precipitates at high salt concentrations by attaching to the suspended coralline particles, and thus precipitates during the centrifugation process. Acidification may alleviate the assay interference by dissolving calcium carbonate-rich coralline particles, thereby limiting the substrate for the DNA to bind.

Following the prior section, turbidity versus control (n=14, R^2=0.427, P=0.112), and salinity versus control (n=14, R^2=0.169, P=0.548) resulted in no significant relationships between any pair of variables.

There was also limited data for the chemistry of the samples since cations and anions were only measured for two of the experiments. No trend or indication was drawn that any of the chemicals in the water are affecting the values obtained (Appendix E, Appendix F).

Table 5: Data for pH-Level Adjustment Experiments of the 5 Beach Samples

		Experiment 1		Experiment 2		Experiment 3	
		CONTROL	ENTEROC.	CONTROL	ENTEROC.	CONTROL	ENTEROC.
BEACH (B.)	**pH**	ΔC_t	ΔC_t	ΔC_t	ΔC_t	ΔC_t	ΔC_t
Kahala B.	**original**	-1.03	0.07	-1.73	-1.89	**-11.841**	**-9.255**
	rinsed	/	/	-1.70	-1.69	-0.016	-1.503
	5	-0.36	-0.66	-1.54	-0.94	-0.242	-0.551
	4	-0.10	-0.73	-0.10	-0.19	-1.338	-1.217
	3	-1.58	-0.75	0.12	-0.29	0.049	-0.398
	2.5	-1.76	-2.40	0.33	-0.66	-0.330	-0.504
Kailua B.	**original**	**-9.34**	**-8.68**	**-3.96**	**-3.89**	**-2.191**	**-3.507**
	rinsed	/	/	-0.81	-1.69	-1.516	-2.286
	5	-2.45	-2.17	-0.03	0.03	**-4.717**	**-8.204**
	4	0.07	-0.23	-0.74	-0.81	-0.927	-0.987
	3	0.06	-0.54	-0.99	-1.00	-0.351	-0.141
	2.5	0.02	-1.08	-0.73	-0.71	0.057	-0.203
Kalama B.	**original**	**-3.94**	**-4.07**	**-9.83**	**-9.93**	**-29.560**	**-10.276**
	rinsed	/	/	**-9.98**	**-10.25**	**-29.560**	**-11.523**
	5	**-4.26**	**-4.17**	-0.75	-0.91	**-29.560**	**-7.178**
	4	-0.79	-1.05	**-5.09**	**-5.22**	**-29.560**	**-11.592**
	3	0.07	-0.72	0.10	-0.05	**-4.478**	**-5.327**
	2.5	-0.55	-1.36	0.05	-0.24	-0.526	-0.872
Sandy B.	**original**	-0.05	-0.42	-2.06	-1.49	-0.372	-1.961
	rinsed	/	/	-1.55	-1.52	-0.384	-1.102
	5	0.15	-0.13	-0.93	-0.57	-0.552	-0.551
	4	0.05	-0.85	-0.93	0.01	0.004	-0.235
	3	-0.15	-0.23	-0.20	0.21	-1.284	-0.846
	2.5	0.01	-0.72	-0.74	0.27	-0.441	-0.273
San Souci B.	**original**	0.01	0.36	-1.17	-1.54	-0.421	-1.418
	rinsed	/	/	-1.30	-2.68	-0.092	-1.272
	5	-0.05	-0.03	-0.29	-0.91	0.027	-0.416
	4	0.36	-0.57	0.17	-0.21	-0.237	0.180
	3	0.23	-0.21	0.21	0.03	-0.246	-0.250
	2.5	0.29	-0.83	0.56	-0.04	0.048	0.102

The pH-level was adjusted from original pH of seawater to 5, 4, 3 and 2 pH for 4 times 100 ml of each beach site and the ΔC_t was calculated for control DNA as well as for enterococci (ENTEROC.). The red values indicate interference.

4.0 DISCUSSION

Earlier studies have demonstrated that not all samples can be analyzed using this rapid method due to inhibition (Weisberg, 2011; Griffith, 2013; Kirs, 2015). A study conducted by a team of scientists from the Southern California Coastal Water Resource Project (SCCWRP) identified PCR interference in 51.9% of the samples (n=181) collected from southern California coastal waters (Griffith, 2013). Another study conducted in Hawai'i showed 70% compromised samples (Kirs, 2015). The source of assay interference is not known, but compounds interfering with the DNA amplification were implicated (Kirs, 2015).

Inhibition can occur when high-molecular-weight compounds in the source water, for example complex carbohydrates, combined with metal ions to sequester nucleic acids from polymerases, prevent amplification (Nobel et al., 2010). To approach inhibition issues in analysis of water samples, the use of DNA extraction kits, dilution, or adding BSA could be helpful (Noble et al., 2010). This can address the interference when it relates to PCR inhibition, but not when the DNA is lost during the rapid DNA extraction process. While the health department could fall back on the culture method for these studies, it was recommended that additional research should be performed to identify ways to correct or eliminate the effects of assay interference (Weisberg, 2011).

This study indicates (Objective 1) that the interference is a result of the DNA loss during the DNA extraction step and not due to the inhibition of PCR reaction. Following up on that finding, in the next part of the trials verified that lowering the pH level of the seawater removed the issue of DNA loss in most samples.

Although the data set of samples which exhibited PCR interference was relatively small (n=10), overall less DNA was lost. With this extra step before filtration, the modified version of the method improves the percentage of samples in which concentration of microorganisms, such as enterococci, can be determined using the rapid method. Monitoring beach water quality will be more efficient and provide greater protection efforts for residents and visitors in Hawai'i.

Commercial DNA extraction kits are effective in alleviating issues with tests interference. Analyses of 110 coastal samples collected in Hawai'i did not identify a single sample where a ΔC_t shift would have indicated 10-fold underestimation of seeded control DNA when commercial DNA extraction kits were used. The added processing time leads to health warnings being posted too late in the day to adequately protect swimmers and the extra laboratory steps, such as spin-column based purification, utilized by commercial extraction kits can add to measurement error (Noble et al., 2010).

Enterococci are frequently isolated from subtropical and tropical soils, where their densities can reach up to 10^4/g soil (Byapanahalli et al, 2012). Therefore, better sewage specific water quality indicators are needed for the state of Hawai'i. In this regards, human-associated *Bacteriodes* assays could be important to incorporate into beach monitoring programs once the epidemiological studies have identified the link between the concentrations of this organisms in the water and the disease rate (Kirs, 2016).

Each year over 5 million visitors enjoy the beaches of Oahu (2015 Annual Visitor Research report). As swimming in contaminated or uncontaminated water would make a difference in ruining or not ruining someone's vacation, application of rapid tests is extremely important in regards to the health of our visitors, as well as residents.

Therefore, application of quicker methods is highly desired in the state of Hawai'i. This study is a step forward in applying these tests for waters that are being used recreationally.

The Department of Health is responsible for monitoring beaches in the State of Hawai'i, but if their budgets are limited, which does not mean that daily application of these methods would not be feasible. Since protection of our visitors is of interest to our tourism industry, hotels and others could step in to support funding of these types of beach monitoring programs, so our beaches would remain a safe place for anyone to use.

One of the questions remaining is whether the new methods can be implemented in a manner that allows for same-day health warnings. While qPCR can theoretically be accomplished in less than two hours, this does not include the time for many other steps in the process. For example, samples need to be collected and prepared, data analysis and quality assurance checks must be completed, also communication of results to the health officers, and posting takes time. Nevertheless, when samples are collected early in the morning and transferred directly to the lab for the analyses, beaches could be electronically posted by 11:00 AM latest by trained sampling and analyses teams (Kirs, personal communications).

5.0 CONCLUSION

This study demonstrates that the assay interference is a result of DNA loss during the extraction process, and not due to the inhibition of PCR reactions, as suspected. The DNA attaches to particles present in seawater, which are centrifuged to the bottom of the test tubes and therefore do not reach the final dilution before processed in the qPCR real-time machine. This can lead to target underestimation and therefore to false-negative results, for example keeping a beach open that should be closed. Since interference can be caused by a range of physical, biological and chemical mechanisms, it is difficult to determine the issue for all qPCR methods in their different fields of possible application.

As shown in these experiments, interference can be eliminated by changing the pH level of the beach water sample to a lower value between five and three pH. The water quality testing in Hawai'i is challenging because of the coral reefs, endemic to the islands and the composition of seawater, possibly containing more sodium and calcium carbonate; more research is required in this matter to make further conclusions. Now that we have identified that DNA loss is the issue of the rapid method, further research in comparing siliceous sands to coral sands, which have different concentrations of chemicals, is needed.

We envision that soon state agencies will be able to analyze beach water quality based on the enhanced rapid methods and post electronic beach notifications online as well as at the beach, before most people arrive, to prevent possible sewage-borne illness. More research needs to be conducted to assure a safe environment for all visitors and residents of Hawai'i, and anywhere recreational water users are at risk from related diseases.

APPENDIX

APPENDIX A: Adjusting Control DNA Concentrations Experiments Results for First Run

Sketa Concentration	High 0.1µg/ml	ΔC_t	Med. 0.01µg/ml	ΔC_t	Low 0.001µg/ml	ΔC_t
Water	20.1	0	25.9	0	26.4	0
Ala Moana Beach	19.9	0.2	23	2.9	26.5	-0.1
Haleiwa Beach	19.9	0.2	23	2.9	26.3	0.1
Sharks Cove	19.9	0.2	23	2.9	26.7	-0.3

APPENDIX B: Adjusting Control DNA Concentrations Experiments Results for Second Run

Sketa Concentration	High 0.1µg/ml	ΔC_t	Med. 0.01µg/ml	ΔC_t	Low 0.001µg/ml	ΔC_t
Water	23.5	0.0	25.6	0.0	30.8	0.0
Ala Moana Beach	22.1	1.3	27.1	-1.4	32.5	-1.7
Haleiwa Beach	22.0	1.4	27.3	-1.7	31.5	-0.8
Sharks Cove	21.8	1.6	26.4	-0.7	30.7	0.1

APPENDIX C: Control DNA in Sample VS Control DNA in Mastermix ΔC_t Results for 3 Trials

	Δ Ct First Run		Δ Ct Second Run		Δ Ct Third Run	
Beach	**Control DNA in Sample**	**Control DNA in Mastermix**	**Control DNA in Sample**	**Control DNA in Mastermix**	**Control DNA in Sample**	**Control DNA in Mastermix**
Kahala	1.5	1.6	-5.7	-0.4	-0.7	1.2
Kailua	-8.0	0.8	-1.1	0.1	-5.0	1.9
Kalama	-7.8	1.8	-7.2	0.3	-1.2	1.8
Sandy	0.3	1.6	-0.3	0.1	-0.1	1.1
Sans Souci	1.3	1.4	-9.7	0.0	1.4	1.9

APPENDIX D:

	pH				Salinity (ppt)				Turbidity (ntu)			
BEACH	**Exp1**	**Exp2**	**Exp3**	**Avg**	**Exp1**	**Exp2**	**Exp3**	**Avg**	**Exp1**	**Exp2**	**Exp3**	**Avg**
Kahala	8.33	8.24	8.48	**8.35**	31.70	30.50	31.10	**31.10**	32.20	28.30	25.70	**28.73**
Kailua	8.32	8.14	8.40	**8.29**	31.30	32.30	33.30	**32.30**	26.70	9.62	33.90	**23.41**
Kalama	8.34	8.23	8.42	**8.33**	32.70	32.90	32.40	**32.67**	23.70	31.10	47.30	**34.03**
Sandy	8.36	8.22	8.35	**8.31**	32.70	33.30	32.10	**32.70**	43.80	11.80	17.90	**24.50**
Sans Souci	8.31	8.20	8.45	**8.32**	32.20	33.40	32.70	**32.77**	11.90	6.50	32.80	**17.07**

APPENDIX E: Chemistry of Water Samples from Second Trial for pH Adjustment Experiment

(mg/L)	Chloride	Bromide	Sodium	Potassium	Magnesium	Calcium	$CaCO_3$
Kahala Beach	311.0	57.0	344.0	0.9	3.4	3.5	23.0
Kahala Beach rinsed	53.0	52.0	117.0	0.4	1.9	3.8	17.0
Kailua Beach	134.0	54.0	193.0	0.8	2.2	5.1	22.0
Kailua Beach rinsed	54.0	52.0	118.0	0.4	1.7	6.3	23.0
Kalama Beach	230.0	58.0	279.0	1.5	3.1	4.5	24.0
Kalama Beach rinsed	55.0	56.0	122.0	1.0	2.4	5.8	24.0
Sandy Beach	159.0	54.0	207.0	0.8	3.7	8.4	36.0
Sandy Beach rinsed	56.0	52.0	99.0	0.2	14.6	37.9	155.0
Sans Souci Beach	104.0	55.0	165.0	1.4	2.0	3.2	16.0
Sans Souci rinsed	59.0	56.0	123.0	0.4	2.3	3.8	19.0
Control (water)	**50.0**	**53.0**	**115.0**	**0.2**	**2.1**	**1.6**	**13.0**

APPENDIX F: Chemistry of Water Samples from Third Trial for pH Adjustment Experiment

(mg/L)	Chloride	Bromide	Sodium	Potassium	Magnesium	Calcium	$CaCO_3$
Kahala Beach	371.0	63.0	302.0	5.0	30.7	4.6	138.0
Kahala Beach rinsed	60.0	63.0	109.0	0.0	6.5	4.3	38.0
Kailua Beach	121.0	60.0	142.0	1.0	9.3	4.8	50.0
Kailua Beach rinsed	56.0	62.0	101.0	0.0	6.5	5.1	40.0
Kalama Beach	294.0	63.0	265.0	3.0	19.1	4.8	91.0
Kalama Beach rinsed	56.0	62.0	103.0	1.0	6.4	6.8	43.0
Sandy Beach	151.0	59.0	160.0	2.0	10.5	4.7	55.0
Sandy Beach rinsed	56.0	58.0	101.0	0.0	2.2	5.3	22.0
Sans Souci Beach	135.0	62.0	149.0	2.0	12.3	3.9	60.0
Sandy Beach rinsed	66.0	62.0	108.0	0.0	9.1	3.9	47.0
Control (water)	**2.0**	**1.2**	**2.0**	**0.0**	**0.3**	**0.0**	**1.2**

APPENDIX G: pH Values for all 5Beaches and all 3 Trials from the pH Adjustment Experiment

Sample Site	Experiment	pH 8	pH 5	pH 4	pH 3	pH 2.5
Kahala Beach	**Exp. 1**	8.33	4.90	4.05	3.06	2.51
	Exp. 2	8.34	5.08	4.07	3.08	2.52
	Exp. 3	8.48	5.02	4.00	3.05	2.51
	Average	8.38	5.00	4.04	3.06	2.51
Kailua Beach	**Exp. 1**	8.32	4.90	4.01	3.05	2.47
	Exp. 2	8.42	4.90	3.90	3.08	2.51
	Exp. 3	8.40	5.12	3.97	3.10	2.56
	Average	8.38	4.97	3.96	3.08	2.51
Kalama Beach	**Exp. 1**	8.34	5.07	4.06	3.06	2.51
	Exp. 2	8.43	5.15	4.01	3.04	3.11
	Exp. 3	8.42	4.96	4.05	2.92	2.50
	Average	8.40	5.06	4.04	3.01	2.71
Sandy Beach	**Exp. 1**	8.36	4.80	4.11	3.10	2.50
	Exp. 2	8.42	4.94	4.00	3.06	2.52
	Exp. 3	8.35	4.97	4.01	3.09	2.53
	Average	8.38	4.90	4.04	3.08	2.52
Sans Souci Beach	**Exp. 1**	8.31	4.90	3.92	3.07	2.53
	Exp. 2	8.39	5.06	3.97	3.11	2.51
	Exp. 3	8.45	5.08	4.10	2.93	2.49
	Average	8.38	5.01	4.00	3.04	2.51

LITERATURE CITED

1. Byappanahalli, Yan, Hamilton, Ishii, Fujioka, Whitman, & Sadowsky. (2012). The population structure of Escherichia coli isolated from subtropical and temperate soils. *Science of the Total Environment, 417-418*, 273-279.
2. Cao, Y., Griffith, J., Dorevitch, S., & Weisberg, S. (2012). Effectiveness of qPCR permutations, internal controls and dilution as means for minimizing the impact of inhibition while measuring Enterococcus in environmental waters. *Journal of Applied Microbiology, 113*(1), 66-75.
3. Colford, Schiff, Griffith, Yau, Arnold, Wright, . . . Weisberg. (2012). Using rapid indicators for Enterococcus to assess the risk of illness after exposure to urban runoff contaminated marine water. Water Research, 46(7), 2176-2186.
4. Fujioka, R., Solo-Gabriele, H., Byappanahalli, M., Kirs, M., & Dorevitch, S. (2015). U.S. Recreational Water Quality Criteria: A Vision for the Future. *International Journal of Environmental Research and Public Health, 12*(7), 7752-7776.
5. Griffith, J.F. 2013. U.S. Recreational Water Quality Criteria (2012): A Vision for the Future. March 11-13,2013 Honolulu, Hawai'i.
6. Harwood VJ, Staley C, Badgley BD, Borges K, Korajkic A. 2014. Microbial source tracking markers for detection of fecal contamination in environmental waters: relationships between pathogens and human health outcomes. FEMS Microbiol Rev 38:1– 40.

7. Hawaii officials reopen Waikiki beach after sewage spill. (2015, August 28). *Real Estate Monitor Worldwide*, p. Real Estate Monitor Worldwide, August 28, 2015.
8. IPCC 2014. *Climate Change 2014. Synthesis Report*. Approved Summary for Policy Makers. IPCC Fifth Assessment Synthesis Report. November 1, 2014, SPM-1: 1-40.
9. Jung, A.V., Le Cann, P., Roig, B., Thomas, O., Baurès, E., Thomas, M.F. 2014. Microbial contamination detection in water resources: interest of current optical methods, trends and needs in the context of climate change. *Int J Environ Res Public Health* **17**;11(4):4292-310.
10. Kirs, M., Caffaro-Filho, R., Wong, M., Harwood, V., Moravcik, P., & Fujioka, R. (2016). Human-Associated Bacteroides spp. and Human Polyomaviruses as Microbial Source Tracking Markers in Hawaii. *Applied and Environmental Microbiology*, *82*(22), 6757-6767.
11. Kirs, M. and Fujioka, R. (2015). Evaluation of Rapid qPCR Method for Enterococci with Correlative Assessment for Molecular Markers for Sewage Contamination in Selected Environmental Water Samples from Hawaii. Final Report, p.15, Water Resources Research Center, University of Hawaii at Manoa, Honolulu, Hawai'i, USA.
12. Noble, Rachel T., Blackwood, A. Denene, Griffith, John F., McGee, Charles D., & Weisberg, Stephen B. (2010). Comparison of Rapid Quantitative PCR-Based and Conventional Culture-Based Methods for Enumeration of Enterococcus spp. and Escherichia coli in Recreational Waters. Applied and Environmental Microbiology, 76(22), 7437.

13. Pencheon, D. (2012). People and planet: From vicious cycle to virtuous circle: Overpopulation, poverty, and environmental degradation share common solutions. *BMJ: British Medical Journal, 345*(7864), 9.
14. Soller JA, Schoen ME, Bartrand T, Ravenscroft JE, Ashbolt NJ. 2010. Estimated human health risks from exposure to recreational waters impacted by human and non-human sources of faecal contamination. Water Res 44:4674 – 4691.
15. Strauch, A., Mackenzie, R., Bruland, G., Tingley, R., & Giardina, C. (2014). Climate change and land use drivers of fecal bacteria in tropical Hawaiian rivers. Journal of Environmental Quality, 43(4), 1475-83.
16. Tomlinson, M.S., E.H. De Carlo, M.A. McManus, G. Pawlak, G.F. Steward, F.J. Sansone, O.D. Nigro, R.E. Timmerman, J. Patterson, S. Jaramillo, and C.E. Ostrander. 2011. Characterizing the effects of two storms on the coastal waters of O‘ahu, Hawai‘i, using data from the Pacific Islands Ocean Observing System. Oceanography 24(2):182–199, doi:10.5670/oceanog.2011.38.
17. USEPA 2012 Method 1611: *Enterococci in Water by TaqMan ® Quantitative Polymerase Chain Reaction (qPCR) Assay*. EPA-821-R-12-008; October 2012, Washington, D.C., USEPA.
18. USEPA 2013 Method 1609*: Enterococci in Water by Quantitative Polymerase Chain Reaction (qPCR) with Internal Amplification Control (IAC) Assay*. EPA-820-R-13-005; March 2013, Washington, D. C., USEPA.

19. Wade, T., Sams, E., Brenner, K., Haugland, R., Chern, E., Beach, M., . . . Dufour, A. (2010). Rapidly measured indicators of recreational water quality and swimming-associated illness at marine beaches: A prospective cohort study. Environmental Health, 9, 66.
20. Water Resources Research Center. (2006). *WRRC Bulletin October, 2006*.
21. Weisberg, Stephen B. (2011). Challenges in Implementing New Technology for Beach Water Quality Monitoring: Lessons from a California Demonstration Project. *Marine Technology Society Journal*, *45*(2), 65-73.
22. 2015 Annual Visitor Research Report. Tourism Research staff of the Hawai‘i Tourism Authority (HTA). This report was prepared by Mr. Daniel Nahoopii, Ms. Jennifer Chun, Ms. Minh-Chau Chun and Mr. Lawrence Liu.