Application of a rapid qPCR method for enterococci for beach water quality monitoring purposes in Hawaii: loss of DNA during the extraction protocol due to coral sands.

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Keywords: enterococci, coastal water quality, EPA method 1611, interference, Hawaii

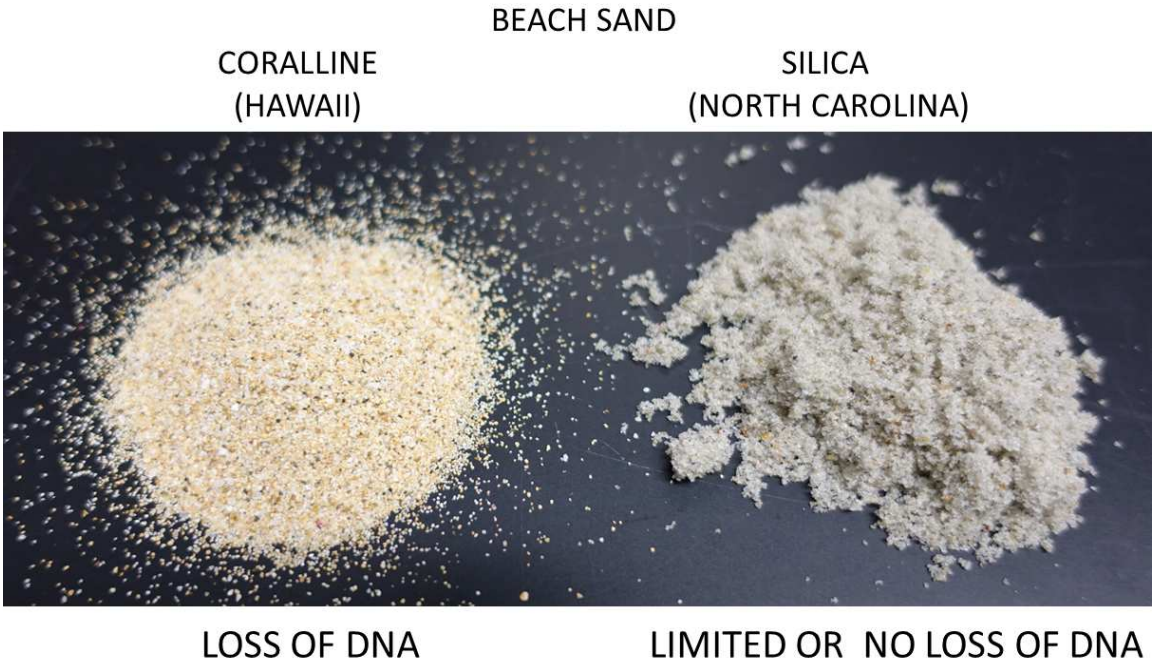
ABSTRACT

Rapid qPCR methods for enumerating enterococci can provide results in a few hours, thereby enhancing public health protection. Analysis of 140 samples collected from 11 beaches in Hawaii for enterococci using EPA Method 1611 revealed that a majority (70%) of samples yielded unusable data using the recommended protocol due to DNA losses during extraction. The DNA loss was correlated to the presence of suspended coralline sand. Acidification of samples alleviated or removed the interference, enabling successful method application. There were significant correlations across the three methods evaluated (Enterolert®/ Method 1600 R^2 = 0.85, Enterolert®/ Method 1611 R^2 = 0.78 and Method 1600/1611 R^2 = 0.82). When samples were not compromised, there was also good agreement among methods for beach management decisions. This study presents a protocol for beach areas with coralline sands, and re-emphasizes the need to use appropriate controls to prevent underestimation of bacterial concentrations at recreational beaches.

Highlights

- Application of EPA Method 1611 for enterococci is hampered in Hawaii.
- DNA is lost during the rapid extraction protocol.
- DNA loss is likely due to coralline sediment present in Hawaiian coastal water.
- Acidification of coastal water samples prior to filtration is recommended.
- Good agreement between beach management decisions based on the different methods.

36 Graphical Abstract



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1. Introduction

Recreational marine water quality in the US is evaluated based on concentrations of enterococci. An association between these concentrations and the incidence of illness in swimmers has been established by several epidemiological studies conducted over past decades (USEPA 2012a). Although the value of enterococci as a health risk indicator has been questioned in subtropical and tropical environments due to the observation that these bacteria can naturalize and grow in warm, nutrient rich extra-enteric environments (Fujioka and Byappanahalli 2003; Byappanahalli et al. 2012), it is likely that the use of enterococci as the indicator organism for marine recreational water quality monitoring programs will continue for the foreseeable future.

Hawaiian recreational water quality standards (HDOH 2014a) are based on the federal Recreational Water Quality Criteria (RWQC) (USEPA 2012a). Therefore, as in other coastal states, marine water quality in Hawaii is evaluated based on enterococci. The Hawaii Department of Health (HI DOH) has also been using *Clostridium perfringens* as a sewage tracer for beach notification purposes (HDOH 2014b). Currently beach advisories are posted online or as physical signs when enterococci concentrations exceed 130 CFU/100ml. This concentration is referred to as the Beach Action Value (BAV) and referred to as such in Hawaii water quality monitoring programs (HIDOH 2017). As water quality cannot be tested daily at each beach due to the limited resources available for the water quality monitoring programs, Hawaii also utilizes brown water advisories. These are published online to warn the public of potential risks from runoff as well as from sanitary sewer and stormwater overflows after significant rain events.

A major practical issue with the application of current laboratory methods for the analysis of enterococci concentrations is that these cultivation-based methods, such as EPA Method 1600, Enterolert®, and others, require ≥24 hour time to get results. Also, confirmation of positive colonies by additional tests is recommended as a quality control (USEPA 2014a) which further extends the time between collection and results. To address this issue, the USEPA approved and released two new molecular methods (EPA Method 1609 and EPA Method 1611)(USEPA 2012b; USEPA 2013) in order to provide the public with near real-time water quality information (Griffith and Weisberg 2010). The results obtained using these methods generally correlate with the traditional culture-based methods for enterococci (Griffith et al. 2009; Noble et al. 2010) and are predictive of gastrointestinal illnesses in beachgoers (Wade et al. 2006; Wade et al. 2008).

Furthermore, as culture-based beach management decisions are frequently no longer significant by the time they are posted, these rapid molecular methods can provide more accurate health-risk based decisions (Colford et al. 2012).

Over eight million tourists visit Hawaii every year (HTA 2014). Application of rapid methods for enterococci would improve Hawaiian water quality monitoring programs and increase the value of beach services. These methods would be particularly well suited to Hawaii as many of the beaches are heavily used and easy to sample. Nevertheless, although there is a well identified need throughout the year for beach water quality information, the implementation of these novel methods in Hawaii is likely to face economic, political, social, and technical challenges.

The objective of this study is to determine whether the new rapid qPCR-based EPA Method 1611 for enterococci could be utilized in recreational water quality monitoring programs in Hawaii. To determine this, we compared traditional cultivation-based to the qPCR-based methods using water samples collected in Hawaii over a one-year period (2013-2014). Related technical issues were identified and addressed in a follow up study (2016-2017). This paper provides a summary of both studies.

2. Methods

2.1 Analyses of one year of coastal water samples: 2013/2014 study

2.1.1 Sample collection

A total of 140 one-liter water samples were collected from 12 beaches on the island of Oahu (Fig. 1; Table 1)) using sterilized plastic sample bottles and either wading to a knee depth or utilizing a telescoping sampling rod. These sites are further referred to as coastal sites. The sampling sites were selected in consultation with the state Clean Water Branch (HI DOH). A total of 11 samples were collected from each site, except for Ala Moana Beach Park where 24 samples were collected and Waimea Bay where only six samples were collected. In addition, 13 samples each were collected from Manoa Stream at Date Street, from the Ala Wai Canal at the canoe ramp, and at the Ala Wai Yacht Harbor (Fig. 1; Table 1). These sites are further referred to as additional sites. All samples were collected between March 21st, 2013 and April 21st, 2014 with monthly sampling events more or less evenly distributed across seasons and conditions. All coastal sites were sampled at knee depth and all additional sites were sampled from shoreline

with a telescoping sampling rod. All samples were collected from top 10-20 cm of water column. Salinity at each site was determined using Pro1030 salinity instrument (YSI; Yellow Springs, OH). All samples were transported in a cooler to the WRRC laboratory at the University of Hawaii at Manoa and analyzed within six hours.

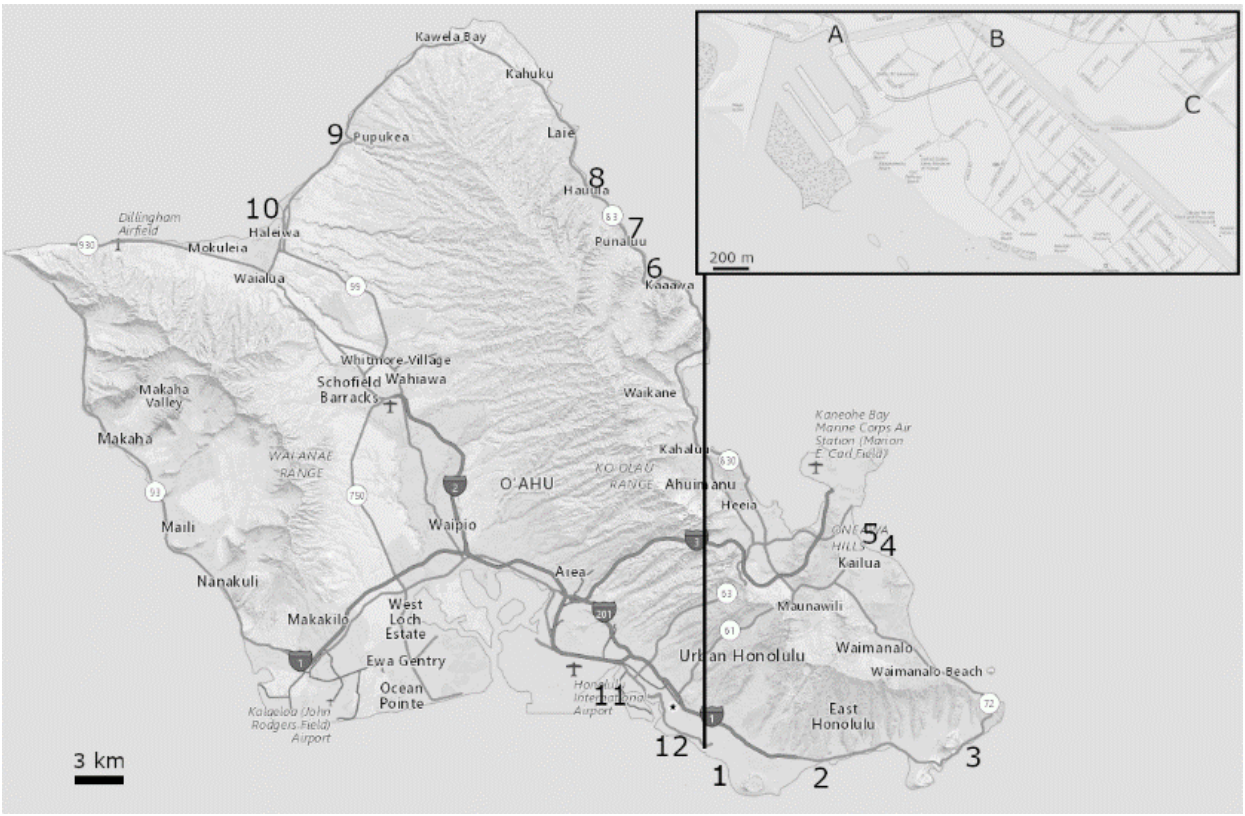

Figure 1. Sample site locations. Coastal sites: 1 - Sans Souci, 2 - Waialae Kahala Beach, 3 – Sandy Beach Pt. No.1, 4 – Kailua Beach Park, 5 – Kalama Beach, 6 – Kahana Bay Beach, 7 – Punaluu Beach Park, 8 – Hauula Beach Park, 9 – Waimea Bay Shoreline, 10 – Haleiwa Beach Park, 11- Keehi Lagoon Pt. X, 12- Ala Moana Beach Park. Additional sites: A – Ala Wai Canal at Yacht Club, B – Ala Wai Canal at canoe ramp, C – Manoa Stream at Date Street.

Table 1. Sample site locations. Tier 3 beaches are not monitored by the Hawaii Department of Health, while Tier 2 beaches are tested five times per year and Tier 1 beaches are tested at least once a week. WQX – Water Quality Exchange site ID number

Site	WQX #	Tier	Main use
COASTAL Sites			
Ala Moana Beach Park - Center	000153	1	swimming, SUP[2]
Haleiwa Beach Park	000171	2	swimming
Hauula Beach Park	000176	3	swimming
Kahana Bay Beach	000230	3	swimming
Kailua Beach Park	000193	1	swimming, kayaking
Kalama Beach	000207	2	swimming
Keehi Lagoon Pt. X	000342	2	kayaking, fishing
Punaluu Beach Park	000177	2	swimming
Sans Souci	000228	1	swimming
Sandy Beach Pt. No. 1	000200	1	swimming
Waialae – Kahala Beach	000214	3	swimming
Waimea Bay Shoreline	000172	1	swimming, surfing, SUP[2]
ADDITIONAL Sites			
Ala Wai Canal - Yacht Club	NA[1]	NA	kayaking
Ala Wai Canal – canoe ramp	NA	NA	kayaking
Manoa Stream (at Date Street)	NA	NA	limited fishing

[1]NA - Not available; [2]SUP – stand up paddle boarding

2.1.2 Culture- and qPCR-based microbiological tests

2.1.2.1 Enterococci by Enterolert®

Concentrations of enterococci were determined in the laboratory using two cultivation-based methods and also by a molecular qPCR-based method (EPA Method 1611). The first cultivation-based method was a defined substrate method (Enterolert®) which was utilized in Quanti-Tray/2000 format according to the manufacturer's protocol (IDEXX Laboratories, Inc.; Westbrook ME). Samples for this test were diluted in Milli-Q® water (Millipore Sigma; Burlington, MA) at a 1:10 ratio prior to analysis and the results were recorded as MPN/100 ml.

2.1.2.2 Enterococci by membrane filtration and mEI (EPA Method 1600)

The second cultivation-based method used was the membrane filtration method, EPA Method 1600 (USEPA 2014a), for which undiluted 100 ml sample portions (coastal sites) or undiluted and 1:10 diluted samples (additional stream sites) were filtered. Filters were placed on Indoxyl-β-D-Glucoside agarose (mEI) plates and incubated for 24 hours at 41.0°C. Colonies ≥0.5 mm in diameter with a blue halo were counted as enterococci.

2.1.2.3 *Clostridium perfringens* by membrane filtration and mCP

In addition, concentrations of *C. perfringens* were determined in 1:10 diluted coastal and additional sites' samples by a membrane filtration-based method (Bisson and Cabelli 1979), which included incubation of filter membranes (GN-6, 0.45 µm pore size; Pall Corp., Ann Arbor, MI) on mCP media in the GasPak™ EZ Anaerobe Pouch System (BD Diagnostics; Franklin Lakes, NJ) at 42°C for 24 hours. The phosphatase test was used to confirm positive *C. perfringens* colonies as indicated by pink, red, magenta color reactions after twenty seconds of exposure to ammonium hydroxide vapors.

2.1.2.4 Enterococci by qPCR (EPA Method 1611)

For the molecular qPCR-based method 100 ml samples were filtered and the filters stored frozen (-80°C), hence the storage time for the filters varied depending from the collection data from one to 14 months. One month after completion of the field studies, the DNA captured on the filters was extracted and analyzed for concentrations of enterococci as specified in EPA Method 1611 (USEPA 2012b), except known concentrations of washed pre-quantified cells of *Enterococcus faecalis* ATCC® #29212™ (10^5 cells per filter) were filtered onto the same filter types using the same protocol, and the filters were extracted in parallel with the field samples and used to make quantification standards as 10-fold dilution series from the original extract. Salmon testes DNA (Sigma D1626), was used as a sample processing control (SPC). Unless otherwise stated, all samples throughout this study, were extracted using identical concentrations of SPC in the lysis buffer as recommended by the US EPA Method 1611. A CFX96™ Real-Time PCR System (Bio-Rad Laboratories, Inc.; Hercules, CA) was used throughout this study as the platform for DNA quantification. In each qPCR run, serially diluted four-point standards (166, 16.6, 1.66 and -0.16 cell per PCR reaction were analyzed), were included in duplicates to estimate concentrations of enterococci and SPC in the extracted samples, including extraction blanks. The lower limit of quantification was 100 cells/and upper limit of detection 100,000 cells /100ml.

160 The lower limit of quantification was determined by 100% detection in the lower dilution used.
161 No template controls, containing no enterococci or SPC DNA, were run in duplicate for each
162 qPCR run. These no template controls remained negative.

163 Concentrations of enterococci were extrapolated from the standard curves using Bio-Rad CFX
164 Manager 3.1 software (Bio-Rad laboratories). Based on the standard curves generated, the
165 efficiencies of the qPCR reactions for enterococci varied from 85.8 to 99.5% (average 93.8%)
166 and for SPC they varied from 85.5 to 95.5% (average 91.8%). The R^2 estimates for the
167 enterococci standards ranged from 0.993 to 0.999 (average 0.997) and for the SPC standard
168 ranged from 0.992 to 0.999 (average 0.996).When the amplification efficiency and/or R^2 for any
169 of the standards did not meet the acceptable criteria (<85%, and R^2 <0.99 respectively) (USEPA
170 2004; Fout et al. 2016), the standards were discarded and the run was repeated with freshly
171 prepared standard. PCR chemistry and conditions were as specified in EPA Method 1611. To
172 identify compromised samples, C_T values for SPC in the Negative Extraction Control (100 ml
173 Milli-Q® water, C_{TNEC}) and sample ($C_{TSample}$) where compared. Samples were considered
174 compromised when ΔC_T (C_TNEC-C_t Sample) for the SPC was equal to or exceeded 3.3 PCR
175 cycles which corresponds to ≥10-fold underestimation of actual concentrations due to
176 interference. While EPA Method 1611 utilizes SPC C_T values to adjust standard curve model, we
177 did not make this adjustment as efficiency of the enterococcus and SPC was different between
178 the standards compared.

179 R^2 and Index Agreement (IA) between cultivation-based and molecular method-based
180 enterococci concentration estimates were determined according to the EPA guidelines for
181 *Alternative Indicators and Methods* (USEPA 2014b) in the Excel spreadsheet format (Microsoft
182 Corp, Albuquerque, NM). Per the guidelines, all samples in which concentrations of enterococci
183 were below the limit of quantification, were excluded when determining the R^2 and IA.

184

185 2.2 Interference: 2016/2017 follow up study

186 2.2.1 Interference - loss of DNA or inhibition. A set of experiments was designed to determine
187 whether the interference associated with coastal water samples in Hawaii is due to the loss of
188 DNA during the rapid extraction step or due to PCR inhibitors. For this purpose, five heavily
189 visited beach sites, Kahala Beach Park, Kailua Beach Park, Kalama Beach Park, Sandy Beach

Park, and Sans Souci Beach Park (Table 1), were selected from the twelve coastal sites. A new set of one-liter water samples were collected at those sites on September 26th, October 14th, and October 20th of 2016 as described above (section 2.1), and transported to the laboratory. In the laboratory two 100 ml portions of each sample were filtered through polycarbonate membrane filters (0.45 µm pore size). One of the filters was extracted as in EPA Method 1611by seeding SPC into the DNA extraction buffer (10 mM Tris-Cl, 0.5 mM EDTA, pH 9.0) as a control for losses during the DNA extraction process and PCR inhibition, while the other filter was extracted using extraction buffer which did not contain SPC. In this case, SPC was added directly into the PCR mastermix as a control for PCR inhibition.

2.2.2 Interference – calcareous coralline sand. To test the hypothesis that calcareous coralline suspended particles were interfering with the DNA extraction, beach sand samples from three coastal sites (Kailua Beach Park, Kalama Beach Park, Sandy Beach Park (Table 1)) were collected. DNA extraction tubes containing silica beads and extraction buffer, as specified in EPA Method 1611.Tubes were seeded with 0.5g, 0.1g, 0.05g and 0.01 g of coralline sand. Each tube was also seeded with an equal concentration of *E. faecalis* ATCC® #29212™ cells (10^6 CFU per tube). Three replicate extraction tubes were extracted and analyzed for SPC and enterococci concentrations according to EPA Method 1611. A set of PowerSoil® Bead Tubes containing no coralline sand were seeded with SPC and *E. faecalis* ATCC® #29212™ cells as above and extracted according to the manufacturer's protocol (MO BIO Laboratories, Inc.; Carlsbad CA).

2.2.3 Interference – silica sand. To compare the effect of coralline and silica sand on DNA extraction efficiency, silica sand was collected from three beaches in North Carolina, one at Pine Knoll Shores on May 22nd, 2017 (Beach access "C") and two at Dogwood Circle Access Area on September 18th, 2017). Silica sand was shipped to the laboratory in Hawaii and analyzed in parallel with Hawaii coralline sand samples. As with the Hawaii samples, the effect of 0.5g, 0.1g, 0.05g and 0.01 g of North Carolina sand on DNA extraction efficiency was investigated. Triplicate tubes were extracted according to EPA Method 1611 for each concentration. For all sand tests (sections 2.2.2 and 2.2.3), the C_T values for SPC and enterococci were determined relative to C_T values identified from samples that contained no sand. EPA Method 1611 extraction and qPCR protocols were used for extraction and determination of Ct values for both targets respectively.

221 2.2.4 Interference – troubleshooting. To test the hypothesis that the interference could be
222 removed by acidifying the samples, five beach sites, Kahala Beach Park, Kailua Beach Park,
223 Kalama Beach Park, Sandy Beach Park and Sans Souci Beach Park (Table 1), were sampled on
224 January 25th, February 13th and March 20th 2017. At each site, a one-liter water sample was
225 collected as above and transported to the laboratory. In the laboratory, samples were split into
226 five 100ml portions and acidified using 6N and 1N hydrochloric acid to pH 5.0, 4.0, 3.0 and 2.5.
227 An unadjusted control was also included for the analyses. Milli-Q® water was used as an
228 extraction control, as clean water should not interfere with the extraction. Each 100ml sample
229 portion, including the controls, was seeded with an equal concentration of *E. faecalis* ATCC®
230 #29212™ cells (8.2×10^6 CFU per sample) prior to the filtration.

231 All samples were filtered, extracted, and analyzed as directed in EPA Method 1611. The C_T
232 values for SPC and enterococci were determined according to EPA Method 1611. ΔC_T was
233 calculated by subtracting sample C_T from the C_T obtained for sample extraction control.

234

235 3. Results & Discussion

236 3.1 Coastal water quality (2013/2014 study)

237 In general, based on the concentrations of enterococci, good water quality was observed at all
238 coastal sites. The Hawaii BAV, 130 CFU/100 ml, was exceeded in five samples (3.6%, n=140)
239 based on the Enterolert® method and in four samples (2.9%, n=140) based on EPA Method
240 1600. In all the samples the BAV was exceeded only twice by both cultivation-based tests
241 concurrently. Both of these samples were collected at Kahana Bay. The water in this bay was
242 frequently brown due to the sediment plume originating from Kahana Stream and poor
243 circulation in the bay. Concentrations of enterococci in Kahana Bay were generally elevated
244 (>35 CFU/100 ml by both cultivation-based tests for seven out of 11 samples) compared to the
245 other coastal sites (Table 2). Concentrations of *C. perfringens* remained below the 50 CFU/100
246 ml threshold (Table 2). Other sites where BAV exceedances were observed were Ala Moana
247 Park Center (two samples by EPA Method 1600 and one sample by Enterolert®), Haleiwa Beach
248 Park (one sample by Enterolert®), and Keehi Lagoon (one sample by Enterolert®). The latter

site is located in a coastal area with mostly industrial land use and was known to be affected by sewage leaks before this study was initiated.

Table 2. Geometric mean and range (in parentheses) of enterococci and *Clostridium perfringens* concentrations (2013/2014 study).

Site	# of samples	Salinity	Enterococci		*C. perfringens*
			Enterolert®	EPA Method 1600	mCP
		ppt	MPN/100 ml	CFU/100 ml	CFU/100 ml
COASTAL SITES					
Ala Moana Beach Park - Center	24	34.7	8.8 (<10-301)	2.8 (<1-180)	5.4 (<10-14)
Haleiwa Beach Park	11	33.0	8.1 (<10-121)	1.9 (<1-56)	5.7 (<10-10)
Hauula Beach Park	11	35.2	6.3 (<10-62)	1.1 (<1-37)	5 (<10)
Kahana Bay Beach	11	24.1	53.9 (<10-389)	30.1 (4-151)	5.7 (<10-20)
Kailua Beach Park	11	34.1	6.0 (<10-20)	1.1 (<1-51)	5.7 (<10-20)
Kalama Beach	11	34.7	7.7 (<10-72)	1.4 (<1-82)	5.8 (<10-30)
Keehi Lagoon Pt. X	11	33.2	16.3 (<10-256)	5.8 (<1-50)	10.1 (<10-96)
Punaluu Beach Park	11	31.5	24.8 (<10-97)	14.7 (<1-63)	5.3 (<10-10)
Sans Souci	11	34.1	9.4 (<10-41)	2.1 (<1-25)	5 (<10)
Sandy Beach Pt. No. 1	11	34.8	6.0 (<10-20)	1.3 (<1-41)	5 (<10-10)
Waialae Kahala Beach	11	34.1	7.0 (<10-31)	4.0 (<1-37)	5 (<10)
Waimea Bay Shoreline	6	33.9	8.9 (<10-41)	1.8 (<1-72)	5 (<10)
ADDITIONAL SITES					
Ala Wai Canal (Yacht Club)	13	26.8	95.8 (<10-7,270)	57.7 (1-3,000)	9.6 (<10-120)
Ala Wai Canal (canoe ramp)	13	23.7	79.3 (<10-10,462)	43.9 (2-3,370)	10.9 (<10-100)
Manoa Stream (at Date Street)	13	4.8	1305 (85-12,997)	790.1(87-10,640)	55.4 (10-490)

255 Table 3. Percentage of samples that could not be analyzed by EPA Method 1611 (USEPA, 2012b) due to the shift of C_T ($\Delta C_T > 3.3$)
256 for the SPC assay.

Site	# of samples	Compromised samples 1611 (%)
COASTAL SITES		
Ala Moana Beach Park - Center	24	79.2
Haleiwa Beach Park	11	63.6
Hauula Beach Park	11	27.3
Kahana Bay Beach	11	72.7
Kailua Beach Park	11	81.8
Kalama Beach	11	81.8
Keehi Lagoon Pt. X	11	81.8
Punaluu Beach Park	11	100
Sans Souci	11	45.5
Sandy Beach Pt. No. 1	11	45.5
Waialae Kahala Beach	11	81.8
Waimea Bay Shoreline	6	66.7
ADDITIONAL SITES		
Ala Wai Canal (Yacht Club)	13	0
Ala Wai Canal (canoe ramp)	13	7.7
Manoa Stream (at Date Street)	13	0

3.2 qPCR – EPA Method 1611 (2013/2014 study)

A high percentage (70.0%) of coastal samples could not be analyzed for enterococci using rapid molecular EPA Method 1611 (USEPA, 2012b) as indicated by the C_T shift ($\Delta C_T > 3.3$) observed for SPC (Table 3). When the samples were further diluted 1:10 in the extraction buffer to 1:50 final dilution of crude DNA extract (USEPA, 2012b), 67.9% of samples remained compromised (three samples improved). This is in contrast to a study conducted on the U.S. mainland where dilution decreased the proportion of compromised samples from 40% to 6% (Haugland et al. 2016), likely indicating the presence of a different type of interference. Compromised samples were not associated with a single beach, but varied between the beaches from 27.3% to 100% (Table 3). From the samples that could be analyzed using EPA Method 1611, only four contained enterococci at concentrations which exceeded the quantification limit of the assay. Therefore reporting beach water quality information based on the protocol as outlined currently in EPA Method 1611 for Hawaiian coastal sites was mostly impossible.

A large proportion of coastal samples could not be analyzed due to the analytical issue and/or exhibited good water quality, hence did not contain measurable numbers of enterococci. Therefore, in order to facilitate comparison between the cultivation-based and molecular methods, a set of 39 samples from additional sites (Ala Wai Canal and Manoa Stream) collected during the same study period, were analyzed. The salinity of those samples was lower compared to those from coastal sites, averaging from 4.8 to 26.8 ppt depending on the site (Table 2). Concentrations of enterococci exceeded BAV in 56.5% and 53.8% of samples analyzed using Enterolert® and EPA Method 1600 respectively (Table 2). In contrast to the coastal sites, only one of these samples (2.3%, n=39) was compromised, as indicated by the C_T shift ($\Delta C_T > 3.3$). DNA losses seen in samples from coastal sites did not appear to be related to salinity, as 73% of the samples collected from the coastal Kahana Bay site, a site with salinity comparable to the Ala Wai Canal sites, were compromised. These findings suggested that some factor, other than salinity, was compromising the method application in the coastal water samples.

Although a high percentage of coastal samples were compromised or did not contain detectable levels of enterococci, we were able to compare enterococci concentration estimates delivered by different methods when the additional set of 39 samples was analyzed. There was good agreement between the two cultivation-based methods (n = 90; $R^2 = 0.847$; IA = 0.93) as well as

between rapid molecular EPA Method 1611 and EPA Method 1600, and Enterolert® (n = 32; R^2 = 0.820; IA = 0.88) and (n = 35; R^2 = 0.777; IA = 0.84) respectively. Furthermore, in most cases there was also good agreement between the beach management decisions that would be made using results of the three methods tested (Table 4). Collectively our data suggests that rapid EPA Method 1611 can be utilized in Hawaii only when the source of interference has been identified and addressed, as the assay has limited use when over 2/3 of samples cannot be analyzed due to the interference.

Table4. Agreement (%) of beach management decisions based on the Enterolert®, EPA Method 1600, and EPA Method 1611. Hawaii BAV of 130 CFU/100ml was used as a criterion for all the comparisons.

		1600				1611				1611	
		close	open			close	open			close	open
Enterolert®	close	68	5	Enterolert®	close	11	33	1600	close	40	9
	open	7	20		open	0	56		open	11	40

3.3 Source of the interference (2016/2017 study)

To identify the source of the interference, experiments were conducted to detect whether the DNA extraction or amplification protocols were compromised. Six out of fifteen samples (40%) were compromised when the SPC was seeded into the extraction buffer according to the protocol outlined in EPA Method 1611 (Table 5). When the SPC was seeded directly to the PCR reaction as part of the mastermix, none of the samples were compromised. This experiment demonstrated that most of the interference observed was not due to the inhibition of PCR reactions, but rather to compromised DNA extraction. We speculate that bacterial DNA binds and pellets with suspended calcareous coralline particles during the rapid DNA extraction procedure. Furthermore, Hawaiian beach sand appears to be rich in coralline material as indicated by high $CaCO_3$ content compared to many other tropical and subtropical areas (Pando et al. 2012). Suspension of coralline beach sand by wave action is the most likely source of this material in the water column. As both rapid methods for enterococci, EPA Method 1609 and 1611, utilize identical DNA extraction protocols, the interference is likely to be associated with both methods.

Table 5. Comparison of ΔC_t measurements for SPC. SPC was seeded either into DNA extraction buffer as extraction and inhibition control or into the PCR mastermix as inhibition control. Bold

314 indicates samples which would have yielded ≥10 fold underestimation of the target DNA. Water
315 samples were collected on three different dates (September 26th, October 14th, and October 20th
316 of 2016) from five popular beach sites.

Site	Δ C_t Date 1		Δ C_t Date 2		Δ C_t Date 3	
	SPC in Sample	SPC in PCR Mastermix	SPC in Sample	SPC in PCR Mastermix	SPC in Sample	SPC in PCR Mastermix
Kailua Beach Park	**-8.0**	0.8	-1.1	0.1	**-5.0**	1.9
Kalama Beach	**-7.8**	1.8	**-7.2**	0.3	-1.2	1.8
Sans Souci	1.3	1.4	**-9.7**	0.0	1.4	1.9
Sandy Beach Pt. No. 1	0.3	1.6	-0.3	0.1	-0.1	1.1
Waialae Kahala Beach	1.5	1.6	**-5.7**	-0.4	-0.7	1.2

317

318 As calcareous coralline particles were the suspected source of the interference, experiments were
319 conducted with coralline beach sand collected from three beaches in Hawaii and silica sand from
320 three beaches in North Carolina. Coralline sand caused strong interference at all concentrations
321 seeded, while silica sand caused interference only at the two highest concentrations seeded (0.5 g
322 and 0.1g per 100 ml) (Fig. 2). This indicates that silica sand is less likely to cause interference,
323 especially since the two highest concentrations tested are probably not typical for coastal water.
324 As the interference appears to be linked to coralline sand, we believe that the interference can
325 compromise the method application in other locations having such sand. In this regard, a recent
326 epidemiological study conducted at Boqueron Beach, Puerto Rico found that a high proportion of
327 their samples were compromised and could not be analyzed using qPCR (USEPA 2009).
328 Coralline sand could have interfered.

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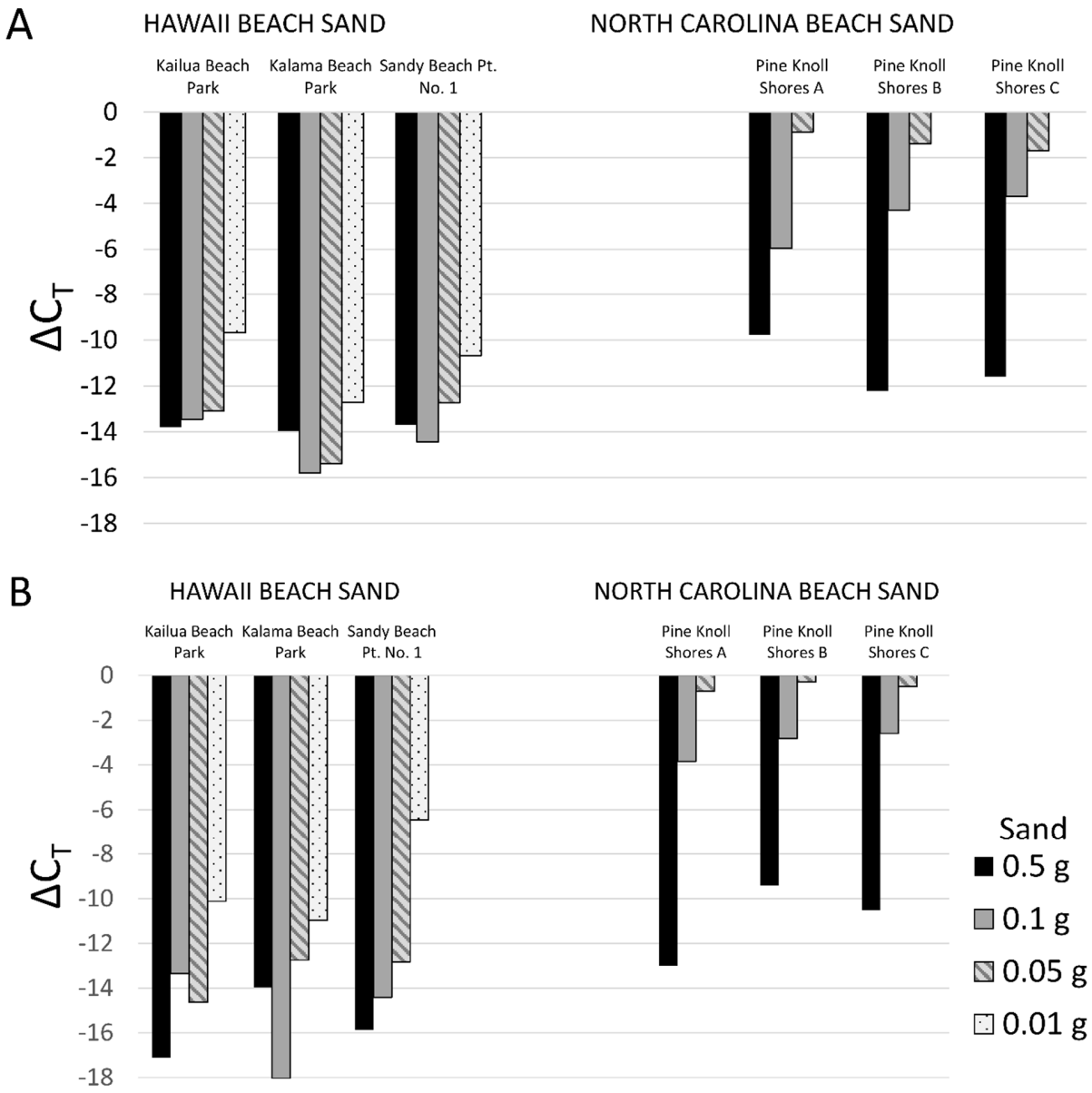

Figure 2. Average ΔC_T (C_TNEC-C_T Sample) as an indication of loss of enterococci (A) and SPC (B) DNA during the rapid DNA extraction process. Different concentrations (0.5, 0.1, 0.05 and 0.01 g) of coralline sand (Hawaii) and silica sand (North Carolina) were added.

3.4 Troubleshooting

As suspended calcareous coralline sand particles were identified as the presumed source of interference in Hawaii coastal samples, experiments were conducted to identify whether acidification of samples with hydrochloric acid could be used to enhance DNA recovery. There was a significant correlation between the pH adjustment and ΔC_T for enterococci (n=35, R^2=0.406, p<0.001) and SPC (n=31, R^2=0.55, p<0.001), indicating that acidification of samples was effective in improving DNA recovery. Where six out of 15 samples (40%) were previously compromised when analyzed for enterococci and seven out of 15 samples (47%) were previously compromised when analyzed for SPC as indicated by a C_T shift exceeding 3.3 PCR cycles, acidification was able to reduce the interference to below the 3.3 threshold in all the samples analyzed for enterococci and SPC. Furthermore, in all samples, except one, analyzed for enterococci, the loss of DNA was less than two-fold, as indicated by ΔC_T <1.0. PCR interference was reduced in most of the samples when the pH was adjusted to ≤4.0 (Fig. 3). Lowering pH below 3.0 could potential damage DNA (An et al. 2015) and is probably not recommended.

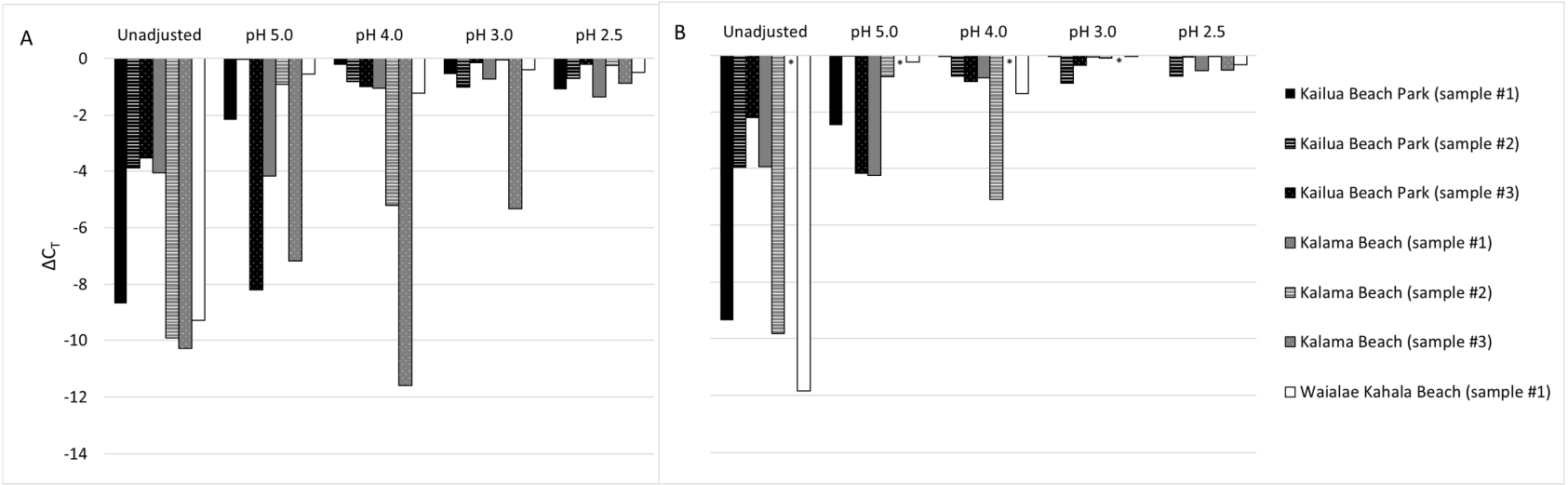

Figure 3. Effect of pH adjustment on the recovery of enterococci (A) and SPC (B) DNA in the compromised samples. Uncompromised samples were not adjusted and were excluded from this analysis. The C_T of all adjusted and unadjusted positive controls did not vary more than 0.51 and 0.09 PCR cycles for enterococci and SPC assay respectively. * indicates samples where ΔC_T could not be determined as no DNA could be amplified in the qPCR reactions (heavy interference).

4. Conclusions

Hawaii's beaches see consistent rough wave action. Wind direction and related wave action vary over the year, altering suspension of sediments. This might explain why interference does not appear to be observed only at certain beaches but was more or less evenly distributed around the island of Oahu. As erosion and resuspension of coralline sand particles can compromise application of rapid methods for enterococci, water samples for analysis using EPA Method 1611 should not be collected close to shore where most of the particles are suspended, and any visible milky plumes, likely containing high concentrations of coralline materials, should be avoided.

In its current form, the application of EPA Method 1611in Hawaii is hampered due to the loss of DNA when the rapid DNA extraction protocol is used. We found this loss of DNA to be correlated with the presence of suspended coralline sand particles. Therefore, it is likely that this issue is not limited to Hawaii and needs to be considered in other subtropical and tropical regions having coralline beach sand. Moreover, this study re-emphasizes the need to use appropriate extraction controls as the loss of DNA is sample specific, and can result in the underestimation of bacterial concentrations and related health risk.

5. Acknowledgements

We would like to thank Ms. Martina Frycova and Mr. Edwin Colon for their assistance in sample collection.

This project was supported by the Department of Interior, USGS Grant G11AP20075 administered by the Water Resources Research Center and NOAA Grant NA14OAR4170071 administered by Hawaii Sea Grant Program.

This is contributed paper WRRC-CP-0000-00 of the Water Resources Research Center, University of Hawaii at Manoa, Honolulu.

6. Conflict of interest

None.

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