Water Research 185 (2020) 116280

Contents lists available at ScienceDirect

Water Research

journal homepage: www.elsevier.com/locate/watres

Fecal indicator bacteria, direct pathogen detection, and microbial community analysis provide different microbiological water quality assessment of a tropical urban marine estuary

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ARTICLE INFO

Article history:
Received 10 June 2020
Revised 5 August 2020
Accepted 6 August 2020
Available online 8 August 2020

Keywords:
Fecal indicator bacteria
Bacterial pathogens
Microbial community structure
Water quality
Urban marine estuary

ABSTRACT

Urban marine estuaries are often impacted by microbiological contamination that impairs use and affects human health acutely, while limited is known about microbiological water quality in urban marine estuaries in the absence of reported sewage spills. This study used a tropical urban marine estuary, the Ala Wai Canal in Honolulu, Hawaii, as the model system to compare fecal indicator bacteria (FIB) concentrations, bacterial pathogen profiles, and microbial community structures. The FIB *Escherichia coli* exhibited higher geometric mean 132 CFU/100mL (n=28) than those of enterococci (18 CFU/100mL) and *Clostridium perfringens* (21 CFU/100mL). Amongst the four pathogens targeted by cultivation methods (*Salmonella, Campylobacter, Listeria monocytogenes* and *Vibrio parahaemolyticus*), only was *V. parahaemolyticus* detected and was detected at high frequency. Microbial community analysis through 16S rRNA gene amplicon sequencing also indicated the high prevalence of *Vibrio* in the water. The pathogen detection patterns and microbial community structure showed no significant correlation with FIB concentration profiles. Together, the results highlight the limitation of using traditional FIB in assessing water microbiological quality in the tropical urban marine estuary environment, indicating the need for more comprehensive microbial risk assessment approaches such as direct detection of pathogens.

1. Introduction

Marine estuaries in urban areas are among the most vulnerable water bodies because of the presence of diverse sources of urban contaminants and the elevated contamination potentials. Marine estuaries are partially enclosed coastal brackish waters that transition from upstream freshwater tributaries to the downstream sea, which makes them prime locations for community development (Crowell et al., 2007). Consequently, urban marine estuaries are ubiquitous and present in nearly all coastal communities. These urban estuaries routinely receive from surrounding communities stormwater runoffs that contain relatively low levels but highly diverse set of contaminants (USEPA, 2009), but can also be inundated occasionally by sanitary sewer overflows that introduce high concentrations of pollutants (Lai et al., 2007). Among these contaminants, fecal pathogens, which are highly abundant in sanitary sewage (Cai and Zhang, 2013) and frequently present in urban stormwater runoffs (Ahmed et al., 2019), carry the highest acute public health risks because of infectious disease transmission associated with recreational uses and fishery aquacultures (Jeng et al., 2005).

Currently, water microbiological quality assessment is based primarily on fecal indicator bacteria (FIB), including *E. coli* and enterococci, which was adopted to indicate fecal pollution and for microbial risks assessment because of the technical challenges associated with directly targeting actual human pathogens (USEPA, 2012). Previous studies showed correlation between water FIB levels and GI illnesses among people exposed to the water (Cabelli, 1983), which formed the foundation of the current FIB-based water microbiological quality monitoring approach. *E. coli* is commonly used as a FIB for freshwater (salinity 0-0.5 ppt) whereas enterococci for both freshwater and marine water (salinity 35 ppt) (USEPA, 2012). Since the salinity levels of brackish estuarine waters vary widely and in-between (0.5-35 ppt) and salinity can have significantly different impacts on the survival of different fecal bacteria (Anderson et al., 2005), the relationship between FIB and fecal bacterial pathogens in brackish marine estuary water are expected to differ from those in freshwater and marine environments.

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https://doi.org/10.1016/j.watres.2020.116280
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Another confounding factor is that the FIB *E. coli* and enterococci are now known to widely exist in the environment other than human and animal feces (Byappanahalli et al., 2012a; Jang et al., 2017). *E. coli* and enterococci are particularly abundant in warm tropical environments, including soil (Byappanahalli et al., 2012b; Goto and Yan, 2011b; Zhang and Yan, 2012) and beach sand (Cui et al., 2013). Since environmental FIB can be transported into estuaries, it is possible that their detection in tropical urban marine estuaries may not be reliable indicator of fecal contamination and fecal pathogen risks.

However, few studies have examined the relationship between FIB and actual human pathogens in tropical urban marine estuary environment in the absence of sewage spills. Previous studies in tropical urban marine estuary were limited to the impact of stormwater on FIB abundance (Wiegner et al., 2017), association with antibiotic resistance genes (Ahmed et al., 2018), their spatial variation (Kirs et al., 2017), and decay kinetics (Maraccini et al., 2016). A previous study conducted limited water sampling at the Ala Wai Canal, a tropical urban marine estuary, and detected *Salmonella* in one of the four water samples (Viau et al., 2011). Common human pathogens of fecal origin include *Salmonella, Campylobacter, and Listeria monocytogenes; Salmonella* and *Campylobacter* are two of the most common bacterial causative agents for gastroenteritis illnesses in USA (Scallan et al., 2011) while *L. monocytogenes* is less frequently reported but with a higher mortality rate (Hernandez-Milian and Payeras-Cifre, 2014). A common non-fecal human bacterial pathogen in marine environment is *V. parahaemolyticus,* which is an opportunistic marine pathogen linked to major foodborne outbreaks worldwide (Okuda et al., 1997) and open wound infections and possibly blood infection (septicemia) from the exposure to contaminated water during recreational activities (Johnson et al., 1984). In the United States, reported *V. parahaemolyticus* illness rate increased from 0.34 (116/342) to 0.48 (605/1252) during 1999-2014 (CDC, 2016; Newton et al., 2012).

In addition, the simultaneous investigations on sediment bacteria and overall microbial community are essential in revealing bacteria distribution and evaluate use of FIB in assessing microbial risks in tropical urban marine estuaries. Microorganisms in sediment tend to survive better due to increased nutrient availability (Haller et al., 2009) and protection from environmental stresses (Garzio-Hadzick et al., 2010). Previous studies reported naturalized waterbed sediment community of FIB and pathogens (Fries et al., 2008). Bacteria sedimentation can result from settlement of either planktonic bacteria cells or large particles with bacteria attached to surfaces, and resuspension can occur through sediment disturbance (Boehm and Sassoubre, 2014), which becomes more likely during heavy rainfall (Frey et al., 2015), hydrodynamic processes (Signell and Butman, 1991), recreational activities (Phillip et al., 2009), or engineering agitation (Dortch et al., 2008). Furthermore, the analysis of microbial community structure previously showed successful application in revealing distributions of FIB and pathogens in marine estuaries in addition to natural marine microbiota (Aylagas et al., 2017; Leight et al., 2018; O'Mullan et al., 2018).

This study used the Ala Wai Canal in Honolulu, Hawaii, as a model urban marine estuary to investigate water microbiological quality as indicated by FIB enumeration, direct detection of select human pathogens, and overall microbial community structure. Water and sediment samples were collected and cultivated for FIB (*E. coli*, enterococci, and *C. perfringens*) and four typical human bacterial pathogens (*Salmonella, Campylobacter, L. monocytogenes,* and *V. parahaemolyticus*). The samples were also subjected to total genomic DNA extraction for cultivation-independent microbial community analysis based on 16S rRNA amplicon sequencing. The abundance of FIB and bacterial pathogens were also compared with various environmental physicochemical parameters.

2. Materials and methods

2.1. Water and sediment sampling

Water and sediment samples were collected from five sites along the Ala Wai Canal, an urban marine estuary on the island of Oahu, Hawaii (Fig. 1). The GPS coordinates for the five sampling sites are: Site 1 (21°16′31.9"N 157°49′04.1"W), Site 2 (21°16′50.2"N 157°49′23.7"W), Site 3 (21°17′18.4"N 157°50′10.0"W), Site 4 (21°17′17.5"N 157°49′55.9"W), and Site 5 (21°17′06.8"N 157°49′43.3"W). Three sampling campaigns were conducted in March (15^{th} and 20^{th}), April (12^{th} and 17^{th}), and May (3^{rd} and 8^{th}) of 2018, which were in the wet season of Hawaii islands when rainfalls and urban storm runoffs frequently occurred. The 24-hours antecedent precipitation prior to sampling ranged from 0.25 to 5.21 cm according to the NOAA weather station MANOA LYON ARBO 785.2 (https://www.ncdc.noaa.gov/cdo-web/datasets/GHCND/stations/GHCND:USC00516128/detail), which is located in the upstream Manoa watershed.

Each sampling campaign used a two-tier approach, including a primary sampling event that determined the presence or absence of four pathogens and a secondary sampling event that followed up within a week for pathogen confirmation and quantification. All water and sediment samples from the five sites were subjected to the primary pathogen detection. When a positive detection of certain pathogen was reported, the secondary sampling event took place at the specific sampling site(s), and the samples were used for quantification of the specific pathogen(s) only. For the primary and secondary sampling events, water samples (four and eight liters, respectively) were collected using sterile polypropylene bottles from water surface near the canal bank. A sediment sampler, which was sterilized by 70% ethanol and rinsed with sterilized deionized water between samplings, was used to collect 250 g of sediment from the sediment/water interface, and the samples were placed in sterile plastic containers. The water and sediment samples were stored on ice and in dark condition and transported to the laboratory for immediate processing (i.e., within six hours).

2.2. Physicochemical parameters

During both primary and secondary sampling events, on-site measurements were performed for pH using an Orion pH meter (Model 290A, USA), and for temperature and dissolved oxygen (DO) using a DO meter (EcoSense DO200A, USA). Water samples were also measured in the laboratory for turbidity (Hach 2100N turbidimeter; Camlab Ltd, Cambridge, UK), salinity (HI 98311, Hanna Instruments; Rhode Island, USA), and total organic carbon (TOC) (TOC-V CPH Total Organic Carbon Analyzer, Shimadzu, Japan). The water samples were filtered through 0.45 um membranes and the filtrates were analyzed for total nitrate (TN) using an ICS-1100 ion chromatography (DIONEX; Sunnyvale, USA).

2.3. FIB enumeration

The common FIB *E. coli* and enterococci and an alternative FIB that is being used in Hawaii (*C. perfringens*) were enumerated for all water and sediment samples collected at both the primary and secondary sampling events. The water samples or their ten-fold dilutions in phosphate buffer saline (100 mL) were filtered through 0.45-μm filters (GN-6-Pall Corporation; Ann Arbor, MI). Sediment samples were first extracted following a procedure previously described (Kingsley and Bohlool, 1981) to release microbial cells before filtration. Briefly, ten grams of sediment samples were mixed in 40 mL of 0.1 M $(NH_4)_2HPO_4$ (pH 7.0) and shaken at 150 rpm for 15 min before the slurry samples were centrifuged at 160 × g

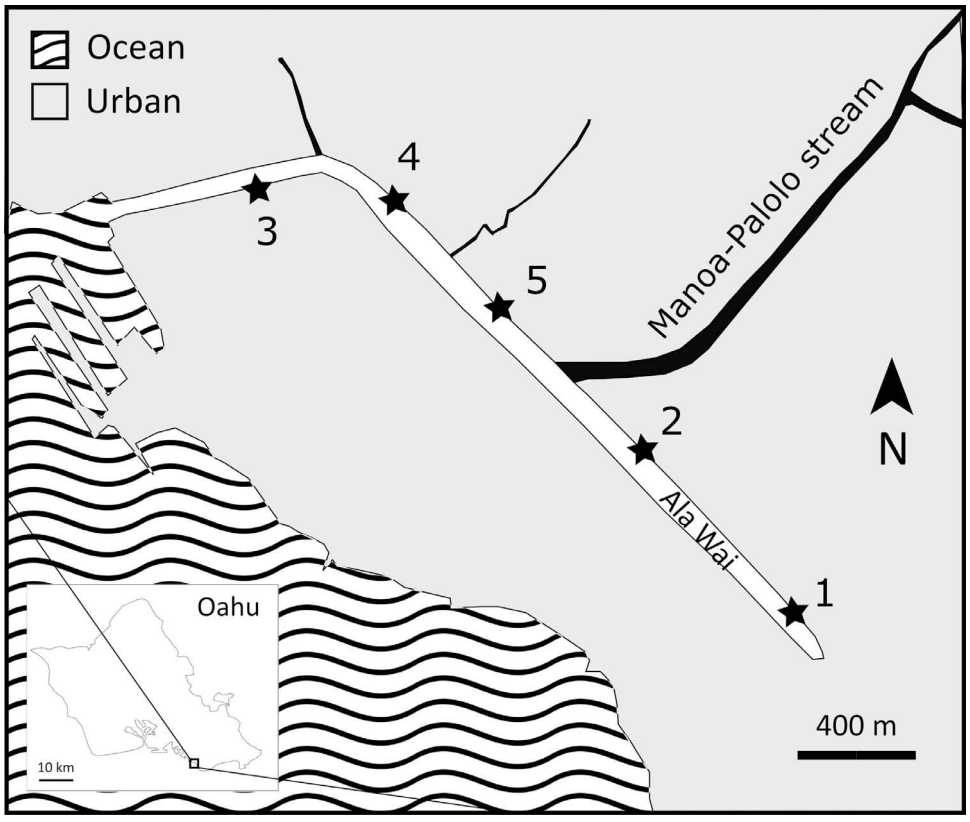

Fig. 1. Water and sediment sampling sites at Ala Wai Canal.

for five minutes. Ten mL of the supernatant were used for subsequent membrane filtration, as described above for water samples. The enumeration of *E. coli,* enterococci, and *C. perfringens* on filters followed the modified mTEC method (USEPA, 2002), the mEI method (USEPA, 2006), and the mCP method (Sartory, 1986), respectively. Sample analyses were performed in duplicate, and the FIB concentrations were reported as CFU/ 100 mL water or CFU/100 g sediment.

2.4. Detection of bacterial pathogens

Water and sediment samples from the three primary sampling events were tested to determine the presence/absence of four different bacterial pathogens, including *Salmonella* spp., *Campylobacter* spp., *L. monocytogenes,* and *V. parahaemolyticus*. Water samples (one liter) were filtrated through 0.45-μm filter (GN-6-Pall Corporation; Ann Arbor, MI), and the filters were submerged in ten mL of enrichment media. Sediment samples (20 grams) were directly placed in 20 mL of enrichment media. The enrichment media and incubation conditions include: tetrathionate broth with iodine and brilliant green at 37 °C for 48 h for *Salmonella* (Ishii et al., 2006b), alkaline peptone water (APW) at 35 °C for 24 h for *V. parahaemolyticus* (Kaysner et al., 2004), buffered *Listeria* enrichment broth base (BLEB) with acriflavin HCl, nalidixic acid, and cycloheximide at 37 °C for 48 hours for *L. monocytogenes* (Hitchins et al., 2017), and Bolton broth with rifampin and polymyxin B sulfate at 37 °C for 48 h in microaerophilic condition for *Campylobacter* spp. (Banting et al., 2016). The occurrence of pathogens was verified by PCR amplification of pathogen-specific target genes as described below. The sampling site(s) where positive detection of certain pathogen(s) occurred were subjected to subsequent secondary sampling for confirmation and quantification of the specific pathogen(s) only.

2.5. Quantification of bacterial pathogens

Since only was *V. parahaemolyticus* detected in water and sediment samples from the primary sampling events, five-tube most probable number (MPN) test for *V. parahaemolyticus* was conducted. For water samples, 1 L, 100 mL, and 10 mL of samples were filtered as described above, and the resulting membrane filters from each of the three volume levels were submerged in five tubes of 10 mL APW enrichment medium. For sediment samples, 20 g, 2 g and 0.2 g of each sample were inoculated to APW enrichment medium. After incubation at 35 °C for 24 h, MPN tubes were subjected to PCR detection of the *V. parahaemolyticus* target gene, and the pathogen concentration was calculated as MPN/L for water samples and MPN/1000g for sediment samples.

2.6. PCR verification of pathogen-specific target genes

The enrichment cultures (from both the detection and MPN quantification experiments) were first extracted using a boiling method to release total genomic DNA. For *V. parahaemolyticus, Salmonella* spp., and *Campylobacter* spp., 1 mL of enriched cultures were centrifuged at 10,000 ×g for 1 minute, and the cell pellets were resuspended in 100 uL of 10mM NaOH. The resuspensions were boiled at 100 °C for 10 minutes and centrifuged at 13,000 x g for 10 minutes, and the supernatant was subjected to ten-fold dilutions in sterile water, which were used as templates for PCR verification. For *L. monocytogenes*, 50 mM NaOH was used for extraction, and the suspension was neutralized to pH 7 with 1 M Tris-HCl. The extraction procedures for genomic DNA were verified using lab reference strains for the target pathogens. The target marker genes for the different bacterial pathogen bacteria and the PCR primers used are shown in Table 1. The PCR products were separated by electrophoresis on 1.5% agarose gel with precast GelRed (Biotium;

Table 1
Target human pathogens and PCR assays for molecular detection.

Target group (gene)	Size (bp)	Primers	Oligonucleotide sequence (5' – 3')	Reference
Salmonella (*invA*)	389	Salm3	GCTGCGCGCGAACGGCGAAG	(Cocolin et al., 1998)
		Salm4	TCCCGGCAGAGTTCCCATT	
V. parahaemolyticus (*gyrB*)	148	VP-F	TGAAGGTTTGACTGCCGTTGT	(Cai et al., 2006)
		VP-R	TGGGTTTTCGACCAAGAACTCA	
C. jejuni and *C. coli* (*cadF*)	183	cadF-F	ACATTATGGCGCGGGTGTAA	(Li et al., 2018)
		cadF-R	GGAGTTGCACGAGTATCAGC	
L. monocytogenes (*hlyA*)	278	hlyA-F	AAACTTCGGCGCAATCAGTGT	(Li et al., 2018)
		hlyA-R	ATTTCCCACTTACGGCAGCA	

Hayward, CA) followed by visualization using a GelDoc imager (Bio-Rad, Hercules, CA).

2.7. Microbial community analysis via 16S rRNA gene amplicon sequencing

Water samples collected during primary and secondary sampling were subjected to total genomic DNA extraction. The water samples (one liter) were filtered as mentioned above, and the filters were stored at -20 °C until DNA extraction. The filters were cut into pieces, and then extracted using PowerSoil DNA Extraction Kit (Qiagen, USA). The V4 hypervariable region of the 16S rRNA gene was amplified by using F515/R806 primers (Bates et al., 2010) linked with the sequencing adapters CS1 (5'-GCTGCGCGCGAACGGCGAAG-3') and CS2 (5'-TCCCGGCAGAGTTCCCATT-3'), respectively. Amplification was confirmed by running PCR amplicons on an electrophoresis and checking for bands with expected DNA size. Library preparation of amplicons, multiplex indexing, and subsequent sequencing on an Illumina Miseq platform was performed by the DNA service facility at University of Illinois at Chicago. An averaged 12,356 paired-end sequencing reads with the length of 153 bp were generated for each sample.

Paired-end sequences reads were quality trimmed and merged using the PEAR software (with parameters: -v 10 -m 300 -n 200 -t 100 -q 20 -u 0.02) (Zhang et al., 2013). Merged reads were denoised and generated unique amplicon sequence variants (ASVs) using the denoise-single command in DADA2 package (Callahan et al., 2016) within QIIME2 (Bolyen et al., 2018) with default parameters. ASVs were aligned to Silva SSU 132 99% database (Quast et al., 2012) and assigned a taxonomy and clustered as taxa using QIIME2 with default parameters. According to taxonomic classification results of 16S sequences, the taxa being identified as the genus *Arcobacter* was considered potential fecal-related human pathogen following the previous review on potential waterborne pathogens (Ashbolt, 2015).

2.8. Data analysis

Concentrations of FIB (CFU/100 mL water and CFU/100 g sediment) and pathogens (MPN/ 1 L water and MPN/1000 g sediment) were log-transformed for subsequent statistical analysis, with no detection (i.e. below method detection limits) numerically represented by 0.9. Correlations between FIB, pathogen (occurrence and quantification), and water quality parameters were based on Pearson's correlation; for data with non-Gaussian distribution as indicated by Shapiro-Wilk's test, Spearman's rank correlation was used. Pathogen occurrence results were assigned with numerical values 0 (no occurrence among tested samples), 0.5 (50% occurrence), and 1 (100% occurrence) prior to Spearman's rank correlation analysis. The correlation between water and sediment were performed similarly and comparison of FIB and *V. parahaemolyticus* levels between water and sediments was analyzed by paired *t*-test, with $P<0.05$ being considered statistically significant, while $P<0.10$ was considered marginally statistically significant.

The taxa that were not classified as prokaryotes were removed prior to relative abundance calculation and data analysis. The taxa that showed relative abundance levels of more than 5% of sequencing reads in at least one of the 28 water samples were considered major populations and was plotted on clustered heatmap by using Seaborn's clustermap function with the UPGMA algorithm. Correlation between microbial community composition, which is indicated by the relative abundance values of taxa, log-transformed concentrations of culturable bacteria, and environmental parameters were determined by redundancy analysis (RDA) using Canoco software (version 5.0). Correlation between relative abundance of fecal pathogen 16S rRNA, culturable bacteria, and environmental parameters was performed using Spearman's rank correlation analysis.

3. Results

3.1. Water quality indicated by FIB

E. coli, enterococci, and *C. perfringens* concentrations in the Ala Wai Canal water were determined by cultivation-based enumeration for all 28 water samples that were collected during both primary and secondary sampling events (Fig. 2A). *E. coli* in the water samples exhibited larger average concentration (geometric mean (GM) of 132 CFU/100mL, range: 2-1340 CFU/100mL) than those of enterococci (geometric mean of 18 CFU/100mL, range: 1-220 CFU/100mL) and *C. perfringens* (geometric mean of 21 CFU/100mL, range: 1-357 CFU/100mL). The GM concentration of *E. coli* in the water samples exceeded the EPA criteria of 126 CFU/100mL, and 17.9% (5/28) of the samples were larger than the EPA statistical threshold value (STV) value of 410 CFU/100 mL. Although the GM concentration of enterococci in the water samples is less than the EPA criteria of 35 CFU/100 mL, 10.7% (3/28) of the samples exceeded the EPA STV value of 130 CFU/100 mL.

To test if different FIB depicts similar water quality scenarios, the concentrations of *E. coli,* enterococci, and *C. perfringens* in individual water samples were compared with each other and against the GM and STV values of EPA water quality standards (Fig. 2B). Although *E. coli* and enterococci concentrations appear to show a positive trend (i.e. higher *E. coli* concentrations correspond to higher enterococci concentrations), the correlation was not statistically significant based on either Pearson's correlation or Spearman's rank correlation (Table S1). Among the 16 and nine water samples that exceeded the GM water quality standards for *E. coli* and enterococci, respectively, only did eight water samples exceed both. Among the five and three water samples that exceeded the STV water quality standards for *E. coli* and enterococci, respectively, only did two water samples exceed both. Correlations between *C. perfringens* and the two common FIB were even weaker than that between *E. coli* and enterococci (Table S1), which is corroborated by the observation that the one single sample with *C. perfringens*

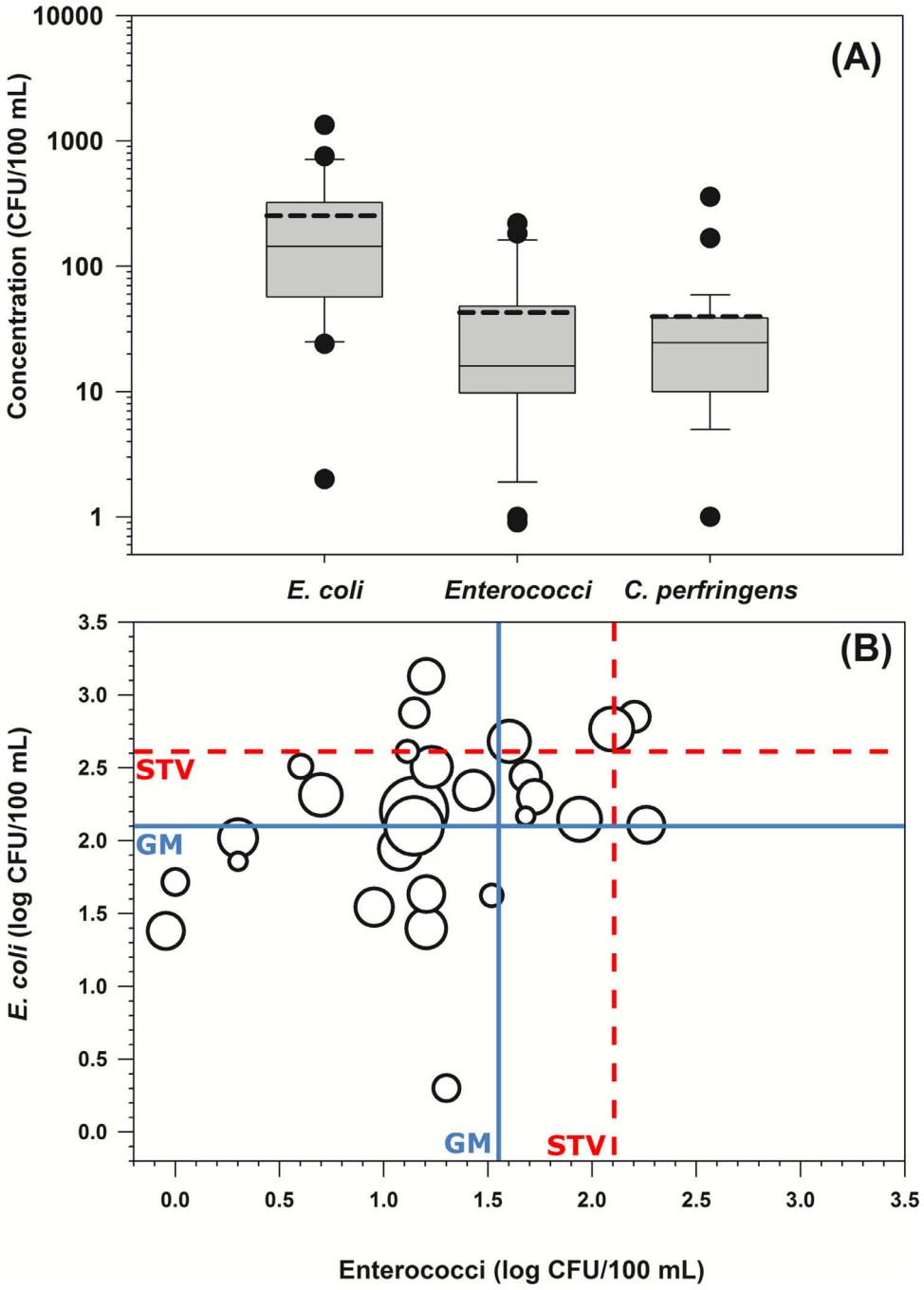

Fig. 2. Concentration distribution of *E. coli*, enterococci, and *C. perfringens* in Ala Wai Canal water samples (A), and bubble plot of *C. perfringens* concentration with respect to enterococci an *E. coli* concentration in the water samples (log CFU/100 mL) (B). The solid lines in the boxplot (A) are lower quartile, median, and upper quartile, while the dashed lines represent geometric means. The solid and dashed lines in (B) indicate the recommended GM and STV water quality criteria for *E. coli* (126 and 410 CFU/100mL) and enterococci 35 and 130 CFU/100mL).

level larger than 50 CFU/100 mL did not exceed either STV standards of *E. coli* and enterococci (Fig. 2B).

3.2. Fecal bacterial pathogens

The occurrence of four different fecal bacteria pathogens, including *Salmonella* spp., *Campylobacter* spp., *L. monocytogenes*, and *V. parahaemolyticus* in the Ala Wai Canal water were determined for the 15 water samples that were collected during the primary sampling events and compared with FIB concentrations (Fig. 3). No *Salmonella, Campylobacter*, or *L. monocytogenes* was detected in any of the water samples, including the water samples showing high levels of fecal indicator enterococci and *E. coli* concentrations. In contrast, *V. parahaemolyticus* was prevalent in the Ala Wai Canal water, with positive detection in 77% (10/15) of the water samples. None of the FIB (*E. coli*, enterococci, and *C. perfringens*) showed significant correlation with the occurrence of *V. parahaemolyticus* (Table S2). Subsequent secondary sampling detected *V. parahaemolyticus* in 100% (10/10) of the water samples. Among the ten positive samples, *V. parahaemolyticus* concentrations ranged from 1.0 to 34.5 MPN/L, with an average of 9.5 MPN/L. Similar to the primary sampling, no significant correlation between *V. parahaemolyticus* and FIB in water samples was detected (Table S2).

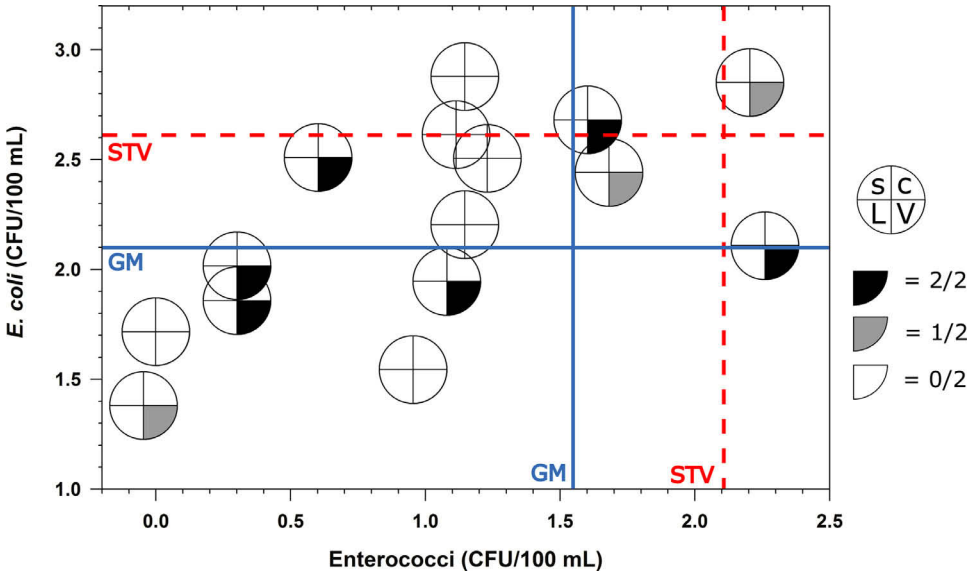

Fig. 3. Pathogen detection (patterns in circles) with respect to enterococci and *E. coli* levels in the Ala Wai water samples. Circles displays presence/absence of pathogens in individual water samples with respect to the GM and STV values of enterococci and *E. coli*. Circle sectors represent pathogens (S = *Salmonella*, C = *Campylobacter*, L = *L. monocytogenes*, and V = *V. parahaemolyticus*) and detection frequencies in replicate samples (e.g. 2/2 = both positive).

3.3. FIB and bacterial pathogens in Ala Wai sediment

To determine whether sediment is a reservoir of FIB and bacterial pathogens in the Ala Wai Canal water, sediment samples were also collected at the same sampling locations and times as the water samples. The detection frequencies and concentrations of enterococci (10/28, range: $10^{1.6}$-$10^{2.8}$ CFU/100g, GM: $10^{1.7}$ CFU/100g), *E. coli* (15/28, range: $10^{1.6}$-$10^{3.1}$ CFU/100g; GM: $10^{2.0}$ CFU/100g), and *C. perfringens* (28/28, range: $10^{1.6}$-$10^{3.3}$ CFU/100g, GM: $10^{2.7}$ CFU/100g) demonstrated no apparent trend or relationship. Similar to the observed patterns of fecal bacterial pathogens in the water samples, no *Salmonella* spp., *Campylobacter* spp., or *L. monocytogenes* was detected in any of the sediment samples, while 40% (6/15) of sediment samples were positive for *V. parahaemolyticus*. Subsequent secondary sampling detected *V. parahaemolyticus* in 43 % (3/7) of the sediment samples, with concentrations ranging from 20 to 114 MPN/1000 g (average 65 MPN/1000 g). No significant correlation between *V. parahaemolyticus* and FIB in sediment samples was detected (Table S2).

Comparison of FIB concentrations between water and corresponding sediment samples showed that the levels of *C. perfringens* and enterococci in the sediment were significantly higher than those in the water column (*t* test, P<0.001) (Table S3), suggesting the potential of sediment as reservoirs of these organisms to the water column. However, no statistically significant rank correlation was observed between the water and sediment compartments; only was a marginally significant positive correlation between *C. perfringens* concentration in the water and sediment compartments was observed (Spearman's ρ=0.35, P=0.07), while no significant correlation was observed for enterococci (ρ=0.08, P=0.68) (Table S4). The *E. coli* level showed no significant difference between the water and sediment samples (*t*-test, P=0.83) (Table S3) and no significant correlation between the paired water and sediment samples (ρ=0.26, P=0.18) (Table S4). *V. parahaemolyticus* concentrations in sediment were not significantly higher than the water body (*t*- test, P=0.41) (Table S3) and no significant correlation of *V. parahaemolyticus* between the paired water and sediment samples was detected (ρ=-0.26, P=0.74) (Table S4).

3.4. Microbial community structures

The 16S rRNA gene amplicon sequencing of the 28 water samples identified a total of 103 different OTUs at the genus level and 88 taxa can be assigned to the genus level. Among them, 27 taxa showed relative abundance higher than 5% in at least one water samples and collectively accounted for majority of sequence reads of the samples (range: 84-100%, average: 91%). These taxa are hence considered major taxa and were used to illustrate the dynamics of the microbial communities (Fig. 4). Microbial communities in the water samples are highly similar, as indicated by the similar composition patterns of the major taxa. However, the clustering of microbial communities was not according to either sampling sites or sampling dates, indicating strong dynamics of the water microbial communities. When ranked by their average relative abundance in all 28 samples, the top five taxa belong to an uncharacterized genus in *Rhodobacteraceae* (21.0%), an uncharacterized genus in *Cryomorphaceae* (14.1%), the Marinobacterium genus (7.5%), the NS5 marine group in *Flavobacteriaceae* (6.0%), and an uncharacterized genus in *Halieaceae* (5.7%). Among the 27 major taxa, 11 could not be classified to previously characterized genera.

There was no apparent correlation between the culturable FIB and *V. parahaemolyticus* levels to the variation of microbial community structure. When the log-transformed level of culturable *E. coli* was tested as the only predictor, significant correlation was indicated (RDA, P = 0.042). However, when all parameters were included in the prediction model, the effect of log-transformed level of *E. coli* became insignificant (RDA, P = 0.596) (Fig. 5). The log-transformed level of culturable *V. parahaemolyticus* did not show the significant correlation to the microbial community variation either (RDA, P = 0.428).

Although no taxa belonging to the genera of *Escherichia, Enterococcus, Salmonella, Campylobacter*, and *Listeria* were detected in the water samples, the genera of *Vibrio* and *Clostridium* were detected. The *Vibrio* genus was detected in 4 of the 28 water samples, with relative abundance range between 1.0% and 17.6%. Occurrence of culturable *V. parahaemolyticus* was also detected in the same water samples (Table 2). While all 3 indicator bacteria were detected

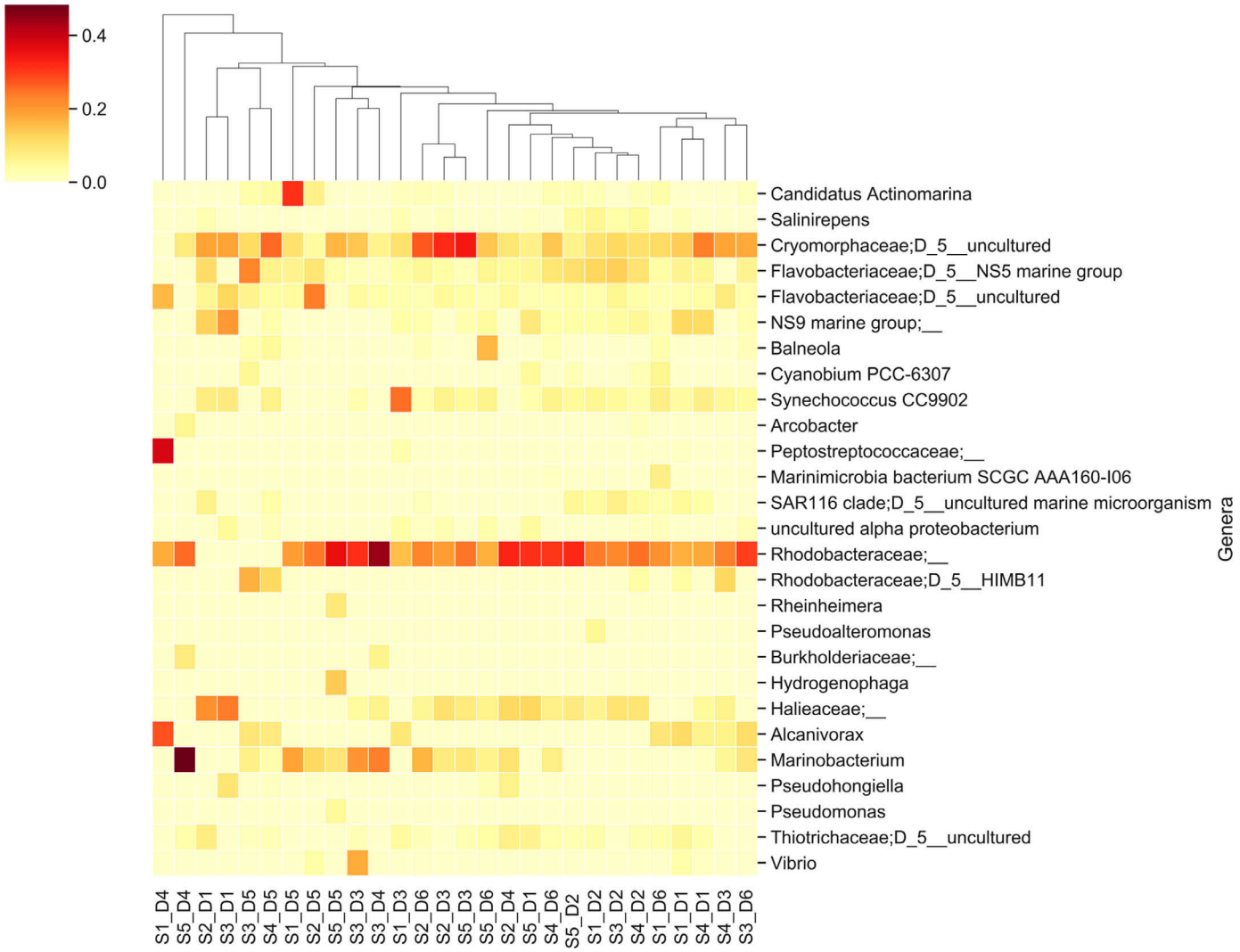

Fig. 4. Distribution of 27 major prokaryotic taxa at the genus level and microbial community clustering in the Ala Wai water samples from different sites and different sampling dates (e.g. S1_D1: Site 1 on Date 1). Sampling dates are: 03/15/18 (D1), 03/20/18 (D2), 04/12/18 (D3), 04/20/18 (D4), 05/02/18 (D5), and 05/07/18 (D6). Those taxa without corresponding taxonomic names at the genus level were indicated by high level names (i.e. family or order) name followed by "_". Color scale represented range of the relative abundance.

Table 2
Four Ala Wai water samples with positive detection of *Vibrio* 16S rRNA through sequencing and culturable *V. parahaemolyticus*.

No.	Sampling site	Sampling date	Relative abundance of *Vibrio* 16S rRNA (%)	Occurrence of culturable *V. parahaemolyticus* [a]
1	1	03/15/2018	3	2/2
2	4	03/15/2018	1	2/2
3	3	04/12/2018	18	1/2
4	2	05/03/2018	4	2/2

[a] The value of occurrence is number of tested samples with positive detection/ number of total tested samples.

by cultivation method, only *Clostridium* was identified through 16S rRNA sequencing. *Clostridium* genus was detected in 1 from 28 water samples and the relative abundance was 1%. In addition, the sequencing was able to detect *Arcobacter* genus in 2 from 28 samples with relative abundance of 1% and 6%.

Relative abundances of *Vibrio, Clostridium*, and *Arcobacter* 16S rRNA showed no significant correlation to log-transformed concentrations of culturable FIB (Spearman's correlation test, $P > 0.05$). Relative abundances of *Clostridium* and *Arcobacter* genes were not correlated to log-transformed concentrations of culturable *V. parahaemolyticus* (Spearman's correlation test, $P > 0.05$). Significant correlation between relative abundances of *Vibrio, Clostridium*, and *Arcobacter* 16S rRNA were not observed either (Spearman's correlation test, $P > 0.05$).

3.5. Fecal bacteria and water environmental parameters

To identify the water environmental parameters that correlate with fecal bacteria abundance in Ala Wai Canal water, physicochemical parameters of the water samples were measured and compared with the FIB and *V. parahaemolyticus* concentrations via correlation analyses. *E. coli* in water indicated significant negative association with temperature and salinity while enterococci indicated significant correlation to only temperature. *C. perfringens* in

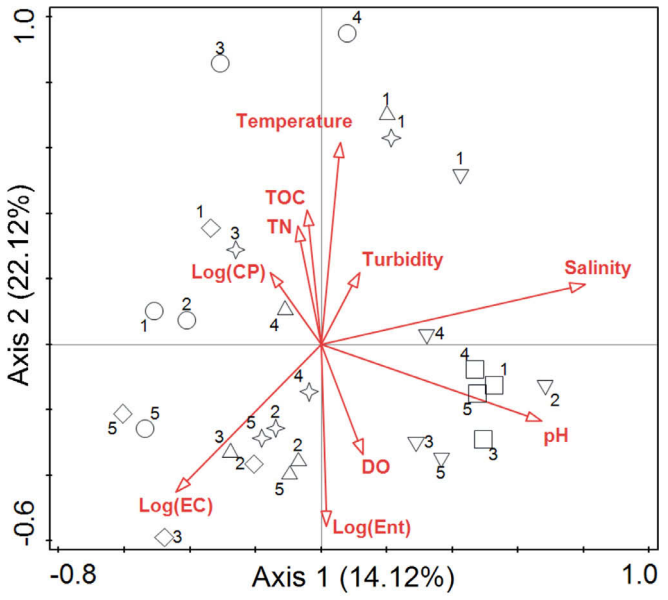

Fig. 5. Redundancy analysis (RDA) ordination plot for the relationship among taxa composition in Ala Wai water samples, environmental physicochemical parameters, and levels of culturable FIB. Symbol shapes indicated samples from different sampling dates (▼: 03/15/2018; □: 03/20/2018; △: 04/12/2018; ⋄: 04/17/2018; ○: 05/03/2018; ✧: 05/08/2018) and the numbers indicated sampling locations. TN: total nitrate; Temp: Temperature; TOC: total organic carbon; CP: culturable *C. perfringens*; Ent: culturable enterococci; EC: culturable *E. coli*.

Table 3
Correlation between log-transformed concentrations of FIB (water) and measured water quality parameters. All values are Spearman's rank correlation coefficients (ρ). Significant correlations are indicated in bold with p-value < 0.05) (n=28).

	Enterococci	*E. coli*	*C. perfringens*
Temp	**-0.50 (p = 0.007)**	**-0.38 (p =0.051)**	0.08 (p = 0.679)
pH	-0.01 (p = 0.969)	-0.06 (p = 0.781)	-0.27(p = 0.16)
DO	-0.08 (p = 0.687)	0.06 (p = 0.756)	**-0.46 (p =0.014)**
Turbidity	-0.12 (p = 0.552)	0.05 (p = 0.797)	**0.44 (p =0.018)**
Salinity	-0.27 (p =0.169)	**-0.50 (p =0.007)**	0.18 (p = 0.355)
TOC	0.088 (p = 0.657)	-0.18 (p = 0.350)	**0.38 (p =0.050)**
TN	-0.16 (p = 0.418)	-0.11 (p = 0.586)	0.10(p = 0.608)

water was significantly correlated with dissolved oxygen, turbidity and TOC (Table 3). There was no significant association between *V. parahaemolyticus* and environmental parameters (Spearman's correlation test, P > 0.05). The relative abundance of *Vibrio, Clostridium*, and *Arcobacter* genes also showed no correlation to water quality parameters (Spearman's correlation test, P > 0.05). In the meantime, the redundancy analysis indicated that salinity were significant primary predictors (p=0.004) of variations in microbial community pattern (Fig. 5).

4. Discussion

E. coli is generally considered more susceptible than enterococci to the osmotic stress caused by high salinity levels, and hence decays faster under such conditions (Evison, 1988). This was an important reason for selecting enterococci as the preferred FIB in marine water (USEPA, 2012). Given their different survival behaviors, the observation of higher levels of *E. coli* than enterococci in the brackish Ala Wai Canal water, which has an average salinity level of 16.63 ppt, contradicts expectations based on the fundamental assumption of the FIB-based water quality monitoring and microbial risk assessment approach. If *E. coli* and enterococci in the brackish Ala Wai Canal water were from the same fecal pollution sources, faster die-off of *E. coli* than enterococci over time is expected to lead to less likelihood of *E. coli* exceeding the water quality standard than that of enterococci. The observation of *E. coli* being more likely than enterococci to exceed water quality standards in the brackish Ala Wai Canal water therefore suggests environmental sources of FIB might have contributed significantly, if not predominantly, to the water samples collected in this study.

Numerous previous studies have shown that *E. coli* (Byappanahalli and Fujioka, 1998; Goto and Yan, 2011a; Ishii et al., 2006a) and enterococci (Byappanahalli et al., 2012b; Byappanahalli et al., 2003; Piggot et al., 2012) are in fact ubiquitous in the environment. The environmental levels of FIB in tropical climates, as in Hawaii, tend to be considerably higher than those in temperate climate regions. For example, *E. coli* concentrations in the soil of Manoa watershed, which drains into the Ala Wai Canal, was reported in the range of 603-1,820,000 CFU/100 g (Goto and Yan, 2011a). Although this level is still significantly lower than that in fecal sources (e.g. municipal wastewater typically contain $10^{5.8}$ – $10^{6.9}$ CFU/100 mL (Reinoso et al., 2008)), it certainly has the potential to be a major contributor of *E. coli*, or even become a dominant source in the absence of actual fecal contamination events. Such environmental sources of FIB undermine the fundamental assumption of FIB-based water quality assessment approach, and potentially limits their utility in water bodies where fecal contamination are not always the most likely sources of the FIB.

The environmental source of FIB in Ala Wai Canal water may also include the underlying sediment. Estuary sediment can be a potential reservoir of microbe due to their protective and nutrient-rich environments (Garzio-Hadzick et al., 2010; Haller et al., 2009). Sediment disturbance could contribute to bacterial levels in overlaying estuarine waters (Perkins et al., 2014). In this study, although no significant correlation of FIB concentrations between sediment and water, enterococci and *C. perfringens* concentration in sediment was usually higher than the overlaying water column. This suggests that sediment could be a potential source of FIB, similar to observations from other previous studies (García-Aljaro et al., 2017; Givens et al., 2014; O'Mullan et al., 2018; Perkins et al., 2014).

The limitation of using the surrogate FIB approach for assessing water microbiological quality is further illustrated by the lack of detection of three major fecal bacterial pathogens, including *Salmonella, Campylobacter*, and *L. monocytogenes*, in all water samples, including those with high levels of FIB where the risks of actual fecal pathogens are expected to be higher. Campylobacteriosis and Salmonellosis ranked the first and second most frequent gastroenteritis (864 and 361 cases) in the 2018 list of notifiable disease by Hawaii State Department of Health (HDOH, 2018). Infected individuals can shed up to 10^9 CFU of *Salmonella* and *Campylobacter* per gram feces (Gopinath et al., 2012; Perez-Perez et al., 1996). A previous study has detected *Salmonella* in Honolulu wastewater with a geometric mean of 89 MPN/100 mL (Yan et al., 2018). Although Listeriosis was generally reported with lower cases than Campylobacteriosis and Salmonellosis (HDOH, 2018) and has lower shedding loads of < 10^4 CFU per gram feces (Sim et al., 2002), asymptomatic healthy individuals could be carriage of *L. monocytogenes* with prevalence varied from 0.2 up to 77% depending on professions and social interactions (Schoder and Wagner, 2012).

A previous study on Ala Wai Canal water also only rarely detected *Salmonella* (1 in 4 samples) and no *Campylobacter* at all (Viau et al., 2011). It might also be possible that the non-detection of the three fecal bacterial pathogens resulted from their faster decay in the Ala Wai Canal water than FIB. While investigations of bacterial decay in estuarine water environment is limited, bacterial pathogens such as *Salmonella* indicated lower persistence

than fecal *E. coli* in marine water microcosms (Chandran et al., 2013). This potential explanation, however, is not supported by the non-detection of the same pathogens in the Ala Wai Canal sediment. Previous studies have shown that following fecal contamination, fecal pathogens tend to have higher concentrations in river (Martín-Díaz et al., 2017), estuary (Perkins et al., 2014), and marine (Givens et al., 2014) sediments than in the corresponding water columns.

The absence of typical fecal pathogen bacteria (*Salmonella, Campylobacter* and *L. monocytogenes)* is contrasted by the almost ubiquitous presence of *V. parahaemolyticus* in the Ala Wai Canal water. *V. parahaemolyticus* is a halophilic organism that inhabits warm marine water and was previously detected in marine estuarine waters (Jesser and Noble, 2018; Johnson et al., 2012). Therefore, it was not surprising that 66% (10/15) of Ala Wai Canal water samples and 40 % (6/15) of sediment samples was confirmed of the presence of *V. parahaemolyticus. V. parahaemolyticus* is a leading cause of foodborne gastroenteritis from contaminated sea foods and can also cause skin infection in open wounds through contact (Scallan et al., 2011), the latter of which is likely to be a more relevant risk factor because of the heavy recreational use of the Ala Wai Canal water. Therefore, its ubiquitous presence and relatively high concentration level (average 9.5 MPN/L) indicate significantly higher public health risks than those imposed by the other three typical fecal bacterial pathogens. Since *V. parahaemolyticus* is not typically of fecal origin, its health risk is not expected to be reflected by FIB even when they are authentic indicators of fecal contamination. Indeed, no correlation between *V. parahaemolyticus* and FIB was detected in Ala Wai Canal water and sediment samples.

The cultivation-based results of the three FIB and four human pathogen results were corroborated by microbial community structures of the Ala Wai Canal water samples revealed by cultivation-independent 16S rRNA gene amplicon sequencing method. No 16S rRNA sequence reads of the *Salmonella, Campylobacter*, and *Listeria* genera were detected. In contrast, *Vibrio* 16S rRNA gene sequence was detected in four water samples with range of 1 to 18% relative abundance. The most abundant genera include a *Rhodobacteraceae* genus ($\mu \pm \sigma$: 21.6±10.9%), a *Cryomorphceae* genus (14.6±7.9%), the *Marinobacterium* (7.7±10.4%), the *Flavobacteriaceae* NS9 marine group (6.3±5.0%), and a *Halieaceae* genus (5.9±6.2%). The *Rhodobacteraceae* family, *Marinobacterium,* and the NS9 marine group are commonly found in marine environments without reported health risks to human, (Cúcio et al., 2016; Naether et al., 2013; Orsi et al., 2016; Pujalte et al., 2014). The *Cryomorphceae* family are commonly found in organic-rich marine and terrestrial water (Bowman, 2014), while the *Halieaceae* family are bacteria inhabiting coastal marine environment (Ivanova and Mikhaĭlov, 2001; Spring et al., 2015).

5. Conclusion

This study showed that different FIB (*E. coli*, enterococci, *C. perfringens*) depicted different water quality pictures in the Ala Wai Canal, a tropical urban marine estuary. Out of four human bacterial pathogens targeted, three common fecal pathogens, including *Salmonella, Campylobacter* or *L. monocytogenes*, were not detected in the water, while *V. parahaemolyticus* was frequently observed and present significant human health risks in the urban marine estuary. Cultivation-independent microbial community analyses detected the *Vibrio* genus but not the FIB or the fecal bacteria pathogens, supporting the high prevalence of *Vibrio* in the water. The detection patterns of the three fecal pathogens showed no significant correlation with the FIB concentrations, while the health risks of *V. parahaemolyticus* as an opportunistic environmental pathogen are not expected to be indicated by FIB. Together, the results highlight the limitation of using traditional FIBs in assessing water microbiological quality in this tropical urban estuary environment. Further research is needed to develop alternative strategies, such as site-specific microbiological water quality standards or direct pathogen detection for comprehensive microbial risk assessment, in order to achieve adequate public health protection.

Declaration of Competing Interest

The authors declare no competing interest.

Acknowledgements

This work was supported by a grant from the National Science Foundation (CBET-1855128 to T.Y.).

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.watres.2020.116280.

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